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A COMPARISON OF AMERICAN STAINS WITH RECENT GERMAN PRODUCTS[1]

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ABSTRACT.—**In 1947, 31 samples of stains of German manufacture were collected in that country, with the coöperation of the U. S. Department of Commerce and one of the American stain manufacturers. They have been compared with current American products by the same tests as used for the samples submitted for certification. Only two proved distinctly superior to the corresponding American dyes, three others as good as the best American samples, four proved entirely unsatisfactory, while seven others were abnormal from the spectrophotometric standpoint although performing satisfactorily when used in the staining procedures by which they were tested.**

During and shortly after World War I, American biologists were faced with a shortage of the accepted German stains or with American stains of questionable suitability. In the period between the wars, however, American dye manufacturers, in close coöperation with a group of biologists especially interested in staining and known as the Biological Stain Commission, have developed standardized stains of high quality. Opportunity to learn how favorably these American stains compare with those of recent German manufacture was lacking until 1947 when a series of 31 German biological stains were collected[2] in Germany and made available for study by the Biological Stain

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[2]These samples were collected by Mr. J. G. Kern, Mr. I. Thornton, and Mr. B. M. Helfaer at the direction of Mr. J. D. Nantz of the National Aniline Division, and distributed by the Office of Technical Services of the U. S. Department of Commerce.

Commission. These stains were subjected to chemical, spectrophotometric, and staining procedures similar to those regularly employed in testing stains submitted by American manufacturers. The results of these tests in comparison with typical American stains form the basis of this communication.

Experimental

The color characteristics of the stains were studied using the Beckman DU Quartz Spectrophotometer at suitable concentrations as determined by current studies on the use of this instrument for routine spectroscopic examination. With each stain, the position and height of the absorption curve were recorded, as well as the mathematical ratio of the color densities at two selected wavelength points on either side of the absorption maximum. These points are generally taken at +15 and −15 mμ of the characteristic maximum, and the ratio of color densities at these points is an expression of the symmetry of the absorption curve near the peak. Since definite points are selected for comparsion, e.g. D520/D550 for a stain with a characteristic peak at 535 mμ, a sample of this stain having an abnormal position of the absorption maximum is also certain to have an abnormal ratio. Hence this ratio is of secondary importance in comparison with the position of the absorption maximum, but has nevertheless been found of use in the optical characterization of stains. The height of the absorption peak at a definite concentration of stain in standard cells (i.e. the absorption coefficient) has been used in this study as a measure of the dye content or strength of the stain, although the validity of this measure has not yet been established with each of the stains.

The staining properties of the dyes have usually been tested either in our laboratories or in collaborating laboratories[3] by the procedures published in Conn's Biological Stains, 5th Edition, pp. 275–310, although for some of the stains additional tests were made to give a better check on their performance.

The following table includes the names of the German stains submitted, the apparent American equivalent, and a comparison of the spectrophotometric data and staining properties of these dyes with other pertinent notes. In examining the data it should be kept in mind that the ranges reported for American samples are

[3]Credit is extended to the following collaborators for their part in these investigations: F. B. Adamstone, E. T. Bell, Sylvia H. Bensley, A. B. Dawson, C. A. Doan, S. I. Kornhauser, Lillian M. Leavitt, R. D. Lillie, B. C. Nesin, O. W. Richards, Sara A. Scudder.

representative but not inclusive. Thus individual samples in a given series of American stains may have been unsatisfactory, although these would not have appeared on the market. Neither can a single sample of a German stain be considered typical; it might be better or worse than the average. (The ranges given for American stains are incidentally not necessarily those indicating approval by the Biological Stain Commission since more extensive investigation is in progress in these laboratories.)

Conclusions

Of the 31 German stains tested, 30 could be directly compared with typical American stains. Of these, 20 were essentially similar in staining and spectrophotometric tests to their American analogs. Three of the German stains, namely *toluidinblau*, *indigokarmin*, and *safranin* were reported as "excellent"; while *azokarmin G* and *B* were found superior to the usual American stain encountered. On the other hand, four of the German stains were considered unacceptable for their staining uses: namely, *rosanilin*, *fuchsin*, *phloxin*, and *pyronin*. The last three of these were also abnormal from the spectrophotometric standpoint. In addition to these, *baumwollblau*, *wasserblau*, *fuchsin*, *Congorubin*, *rose Bengal*, *Sudan III* and *gentian-violett* were also abnormal in their color characteristics, but since these were reported satisfactory for staining, some might have been approved if submitted as American samples. Further details are recorded in the Table.

As a general conclusion, it seems evident that the German stains, as represented by the 31 samples tested, offer no advantage over American stains. The few German stains reported as excellent or superior are more than counterbalanced by important stains which are quite unsatisfactory in the German series. There remains little or no claim for the superiority of German stains which might have existed during and immediately after World War I.

TABLE 1.—COMPARISON OF SPECTROPHOTOMETRIC AND STAINING PROPERTIES OF AMERICAN AND WORLD WAR II GERMAN STAINS

The samples specified as "M.C. No. ——" are the German samples.

Those not so designated represent composite data on typical American samples.

German Name and American Equivalent	Absorption Peak (Mμ)	Ratio of Color Densities*	Color Density† (D_s)	Staining Quality, Acceptability and Remarks
Methylenblau-"stand" M.C. No. 236	665 (water)	D650/D680 = 1.44	193	Typical sample, acceptable.
Methylene blue	663–664	D650/D680 = 1.40–1.60	193–233	
Toluidinblau-"stand" M.C. No. 217	637 (water)	D610/D640 = 0.86	88.0	Excellent staining results. Typical of late American samples. Satisfactory chemically.
Toluidine blue O	625–637	D610/D640 = 0.90–1.10	94.0–110	
Niblau-"stand" M.C. No. 235	644 (50% alcohol)	D620/D650 = 0.81	152	(a) Satisfactory staining. (b) Fair but passable staining. Satisfactory chemically.
Nile blue	637–655	D620/D650 = 0.67–1.22	86.0–186	
Baumwollblau M.C. No. 230	613 (water)	D590/D620 = 0.86	51.0	Staining satisfactory. Decolorization test required more time and alkali. Absorption peak and ratio considerably different from American samples. Not acceptable as "Aniline Blue".
Aniline blue W.S.	592–605	D590/D620 = 1.08–1.20	40.0–67.0	
Wasserblau-"stand" M.C. No. 211	613 (water)	D590/D620 = 0.86	44.0	Staining satisfactory. Weaker than American aniline blues or methyl blues (C.I. No. 706). Decolorization test satisfactory.
Aniline blue	592–605	D590/D620 = 1.08–1.20	40.0–67.0	Absorption peak and ratio atypical. Not acceptable as "Aniline blue".

Kristallviolet-"stand" M.C. No. 227	590 (water)	D575/D605 = 1.04	230	Satisfactory, staining and chemically.
Crystal violet	590	D575/D605 = 1.11–1.12	210–245	
Methylviolett-"stand" M.C. No. 231	582 (50% alcohol)	D575/D605 = 1.50	195	Staining satisfactory. More red than American samples. Acceptable.
Methyl violet	585–587	D575/D605 = 1.33–1.51	200–225	
Gentian violet M.C. No. 233	585 (water)	D575/D605 = 1.48	115	Staining satisfactory. Low color intensity. Not acceptable chemically.
Gentian (Methyl) Violet**	586	D575/D605 = 1.38–1.41	195–215	
Indigokarmin-"stand" M.C. No. 219	610 (water)	D595/D625 = 1.02	36.0	Excellent staining results. Satisfactory chemically.
Indigo carmine	608–610	D595/D625 = 1.03–1.06	37.3–44.0	
Azokarmin B- "stand" M.C. No. 203	511 (water)	D500/D530 = 0.96	23.0	"Azokarmin B" similar in spectrum to G, lower color intensity, but excellent stain. "Azokarmin G" showed strong and brilliant staining, resistant to phosphotungstic acid, stronger than American stains in color. Very satisfactory.
Azokarmin G- "stand" M.C. No. 218	512	D500/D530=0.99	30.0	
Azocarmin G	512	D500/D530 = 0.98–1.00	24.5–25.5	
Saurefuchsin M.C. No. 215	544 (Dilute acid)	D530/D560 = 1.25	64.0	Only passable as connective tissue stain. Satisfactory as Andrade indicator. Excellent and precise staining in Bensley's technic. Satisfactory chemically.
Acid fuchsin	542	D530/D560 = 1.37–1.58	73.0–87.0	

TABLE 1—Continued

German Name and American Equivalent	Absorption Peak (Mμ)	Ratio of Color Densities*	Color Density† (D_s)	Staining Quality, Acceptability and Remarks
Fuchsin M.C. No. 223	552 (50% alcohol)	D530/D560 = 0.82	173	"Fuchsin" satisfactory for T.B., Endo, and Bacteriostatic, unsatisfactory for Feulgen. Not acceptable as either rosanilin or pararosanilin chemically. "Rosanilin-stand" completely unsatisfactory for T.B. staining and Verhoeff's technic. Very poor for Feulgen, Endo, and Bacteriostatic. Typical rosanilin chemically.
Rosanilin- "stand" M.C. No. 206	549 (50% alcohol)	D530/D560 = 0.99	267	
Rosanilin	548–549	D530/D560 = 0.90–0.99	247–260	
Pararosanilin	545–546	D530/D560 = 1.16–1.27	253–263	
Congorubin-"stand" M.C. No. 208	520 (water)	D490/D520 = 0.79	34.0	Staining only fair. Color more like acid fuchsin; does not contrast well with iron hematoxylin. Spectroscopically much redder than American samples. Would not be accepted.
Congo red	498–499	D490/D520 = 1.11–1.12	43.0–63.0	
Erythrosin-"stand" M.C. No. 191	526 (water)	D510/D540 = 0.84	90.0	Staining satisfactory. Spectrum shows a more purple constituent not typical of American samples.
Erythrosin	526–527	D510/D540 = 1.02–1.20	86.0–100	
Neutralrot-"stand" M.C. No. 213	542 (acid 50% alcohol)	D525/D555 = 0.99	118	Staining satisfactory. Most satisfactory for supravital. Not toxic to cells nor any impairment. Typical chemically, high color intensity.
Neutral red	541	D525/D555 = 0.97–1.02	66.0–114	
Phloxin-"stand" M.C. No. 216	525 (alk. 50% alcohol)	D530/D560 = 3.32	76.0	Unsatisfactory staining—deep red, too intense. Spectroscopically too yellow, not typical of American samples. Not acceptable.
Phloxin	545–547	D530/D560 = 1.06–1.36	88.0–140	

Safranin-"stand" M.C. No. 209	532 (50% alcohol)	D515/D545 = 1.01	116	Excellent staining, especially for Flemming triple stain. Chemically typical and acceptable.
Safranin	532–533	D515/D545 = 1.00	130–140	
Pyronin-"stand" M.C. No. 192	545 (50% alcohol)	D530/D560 = 1.35	42.0	Staining fair on G.C. smear, not bright red. Unsatisfactory in Scudder technic; too weak, did not penetrate. Spectroscopically more yellow than Pyronin Y. Weak in color intensity. Would not be accepted.
Pyronin B	554–555	D530/D560 = 0.48–0.60	56.0–118	
Pyronin Y (G)	547–550	D530/D560 = 0.80–1.00	60.0–176	
Bordeaux R-"stand"‡ M.C. No. 204	—	———	—	Staining satisfactory.
Orange G-"stand" M.C. No. 232	480 (water)	D470/D500 = 1.04	34.6	Staining satisfactory. Chemically typical of American samples.
Orange G	476–479	D470/D500 = 1.01–1.06	28.0–39.3	
Eosin-"stand" M.C. No. 224	516 (dilute alkali)	D500/D530 = 1.25	104	Staining satisfactory. Chemically acceptable.
Eosin Y	517	D500/D530 = 0.96–1.10	104–120	
Lichtgrün-"stand" M.C. No. 225	631 (water)	D620/D650 = 1.21	59.0	Staining satisfactory. Chemically acceptable.
Light green	632–633	D620/D650 = 1.18–1.37	70.0–100	
Malachitgrün-"stand" M.C. No. 193	617.5 (water)	D605/D635 = 1.22	207	Staining satisfactory. Typical chemically.
Malachite green	617.5	D605/D635 = 1.22–1.28	180–200	
Brilliantgrün-"stand" M.C. No. 205	630 (50% alcohol)	D615/D645 = 1.02	217	Staining only fair, but passable. Chemically typical of of American samples.
Brilliant green	630	D615/D645 = 1.01–1.03	203–223	

TABLE 1—Continued

German Name and American Equivalent	Absorption Peak (Mμ)	Ratio of Color Densities*	Color Density† (D_s)	Staining Quality, Acceptability and Remarks
Auramin M.C. No. 234	431 (water)	D420/D450 = 1.24	124	Satisfactory staining. Dissolves slowly, needs filtering, slightly oxidized. Spectroscopically typical of American samples.
Auramine O	431	D420/D450 = 1.22–1.27	130–140	
Alizarinrot S-"stand" M.C. No. 229	555, 595 (dilute alkali)	D545/D575 = 1.11	34.0	Satisfactory staining. Typical chemically.
Alizarin red S	556, 595	D545/D575 = 1.09–1.12	35.3–40.6	
Bengalrosa-"stand" M.C. No. 220	551 (alk. 50% alcohol)	D540/D570 = 3.32	64.0	Satisfactory staining. Considerably different spectroscopically from American samples.
Rose bengal	553–558	D540/D570 = 1.10–2.08	70.0–92.0	
Methylorange-"stand" M.C. No. 228	508 (dilute acid)	D490/D520 = 0.92	114	Satisfactory staining. Essentially same spectroscopically as American samples.
Methyl orange	508	D490/D520 = 0.90–0.91	126–130	
Sudanrot III-"stand" M.C. No. 188	515 (benzene)	D490/D520 = 0.84	75.0	Satisfactory staining. Visually and spectroscopically more like American Sudan IV samples.
Sudan III	510	D490/D520 = 0.89–0.90	71.2–83.7	

*See text.

†$D_s = \frac{\log I_0/I}{c \times l}$ = "specific density", where I_0 and I are the intensities of light emerging from the solvent and sample cells respectively, c is the concentration of the dye sample in g. per liter, and l is the length of the light path in centimeters.

**This series refers to samples submitted under the name "Gentian violet" by one of the American manufacturers.

‡Not submitted by American manufacturers.

POLLEN TUBE CULTURE ON A LACTOSE MEDIUM

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ABSTRACT.—**A modified sugar-agar technic for growing pollen tubes is described in which a Van Tieghem cell is used as a moist chamber, and 12% lactose substituted for the normally used sucrose. The advantages offered over previous technics are: (1) direct observation of germination and growth at any stage and for any period of time, (2) easier prevention of bacterial contamination with the long growth periods required to obtain pollen tube divisions, (3) simple method for making permanent slides which are suitable for cytological study.**

During recent years increased use of pollen tubes in the study of cell sensitivity to physiological changes and to the effects of short wave radiation has made it necessary to develop a technic for growing the tubes which is simple, has the least chances of failure, and will allow proper staining and easily made permanent slides that are suitable for cytological study.

Most technics employed at the present time make use of slides coated with a layer of sucrose agar, dusted with pollen, and placed in a moist chamber. The method reported in this paper is a modification of this technic which has certain advantages over that in general usage.

The agar medium is spread on a 22 mm. square cover glass instead of a slide, and the moist chamber is provided in the form of a Van Teighem cell constructed from a glass ring attached to the slide by means of a small amount of vaseline or Canada balsam, and containing several drops of water in the bottom (Fig. 1). The cover glass with agar medium is inverted over the glass ring, using a little vaseline to seal it. In a very short time the air within the Van Tieghem cell becomes saturated, and thus prevents the agar from drying.

The second modification is that lactose is used in place of sucrose as the type of sugar to prepare the medium. As lactose has the same empirical formula as sucrose, and hence the same molecular weight, equal amounts of either sugar give a solution with the same osmotic pressure. The medium used for the growth of pollen of *Tradescantia paludosa* contained 3% agar, 12% lactose, and 0.01% colchicine made up in 25 ml. lots with distilled water.

The relatively high concentration of agar is used because it is easier to spread on the cover glass in a thin even coat. The medium may be applied with a glass rod or a clean finger-tip. Alternatively, the whole cover glass may be dipped in the hot medium, and then the excess allowed to drain by touching the edge of the cover glass to a piece of paper towelling. When the cover glass is dipped in the solution, a 1% agar medium will give a thinner and more even coat. If this method is used, the agar may be removed from one side of the cover glass after it has solidified.

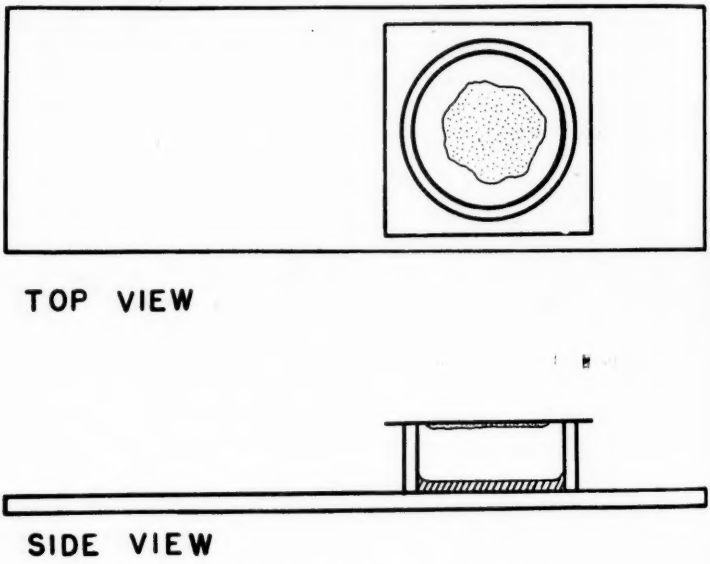

FIG. 1.—Van Tieghem cell adapted for pollen tube culture.

The sugar concentration is higher than that used by most investigators for *Tradescantia* pollen (e.g., see Swanson, 1940), but was chosen after a number of tests because it seemed to give better continued growth, which is essential if the pollen tubes are to develop to the point of the pollen tube division. Lactose was chosen in place of sucrose for two reasons: (1) preliminary tests seemed to indicate that it gave results which were slightly better than with sucrose, and (2) it was much simpler to prevent bacterial contamination with the lactose medium.

The colchicine was added to the solution to inhibit the development of the spindle mechanism. This prolongs the metaphase

period, and thus causes metaphase figures to increase in number on the slide. It also allows the chromosomes to spread out in the long, narrow pollen tube. Ideally, they are strung out one after the other (Fig. 2), though in actual practise this is relatively infrequent.

In preparing cultures with the pollen from *Tradescantia paludosa* the following technic was used. Freshly opened flowers were picked in the early morning, and the pollen dusted on the medium directly from the anthers with the aid of a camel's hair brush. Improved germination and growth were found when the lactose-agar was spread very thinly, an effect also noted by Beck and Joly (1941). This necessitated a minimum time between spreading the agar and completion of the culture in order that the medium remain moist—a maximum of about two minutes.

The thinness of the agar medium used in this method allows the pollen tubes to grow in only one plane. This is of considerable

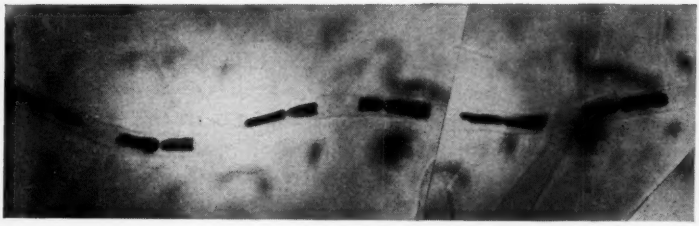

FIG. 2.—Colchicine-inhibited metaphase of the pollen tube division in *Tradescantia*.

importance in the analysis of slides, particularly if photography is required.

Germination percentages of 80% to 95% were normally obtained with this method. On the medium used, the time required for the pollen to reach the metaphase stage of the pollen tube division was in some cases as low as eight to ten hours, but generally was a minimum of about twelve hours. In one lot of 27 cultures, an average of 43 countable metaphases per cover glass was obtained, with the maximum at 121 metaphases.

When the pollen tubes have been growing for 13–15 hours, the cultures may be fixed and permanent slides made in the following manner. The cover glass is removed from the Van Tieghem cell, placed in alcohol-acetic (3:1) for a few seconds, and then inverted on a large drop of acetocarmine on a slide. The preparation may be examined at this point and any failure eliminated. After staining for several minutes, the cover glass is removed with a pair of tweezers, passed through the following dehydration series:

(1) 3 parts 70% alcohol, 1 part glacial acetic acid.
(2) 3 parts absolute alcohol, 1 part glacial acetic acid.
(3) 9 parts absolute alcohol, 1 part glacial acetic acid.
(4) absolute alcohol.

and mounted in diaphane. A prolonged immersion (more than 15 seconds) in absolute alcohol, causes the development of crystals in the medium and makes analysis of the slide difficult or even impossible.

Discussion

One important advantage of this technic is that the growing pollen may readily be observed[1] for any length of time without danger of the culture drying up or the growth being injured in any way. Eigsti (1940) found that pollen tube growth may begin within 15 minutes after sowing. Though varying with the kind of pollen and the concentration of sugar used, with this technic it can be seen that in 10–12% lactose the grains frequently begin to form pollen tubes between two and three minutes after sowing, have mostly germinated in five minutes, and any that do not germinate in fifteen minutes probably never will develop. The method is ideal for class demonstration of cell growth and cytoplasmic streaming. With optimum conditions pollen tubes have grown 500 μ in an hour, and cytoplasmic streaming was measured at 200 μ a minute.

Active streaming in some tubes may continue for as long as 36 hours, but normally when the tubes reach their maximum length, in *Tradescantia* usually 2000 to 3000 μ (though it may be 5000 or more if the tube is very narrow), the end becomes enlarged and frequently bursts, spewing out the nuclear contents.

It is rather interesting that lactose, which is a non-nutrient sugar as far as pollen grains are concerned, can be used with results as good as with the nutrient sugar, sucrose. This shows that for pollen tube growth, which in terms of the size of the original pollen grain is tremendous, external nutrient is not required. Apparently the function of the sugar is solely as a non-toxic substance which regulates the external osmotic pressure to the point where water may be absorbed at a slow rate, so that growth may be achieved without pollen tube bursting.

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[1]With some microscopes, better lighting is obtained by removing the top lens of the condenser, thus increasing its focal length.

THE SELECTIVE DEMONSTRATION OF RED BLOOD PIGMENTS (HEMOGLOBIN AND ERYTHROCRUORIN) IN MICROSCOPIC BALSAM PREPARATIONS[1]

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ABSTRACT.—**Using smears of Amphibian testis, after fixation with a 5% aqueous solution of sulfosalicylic acid, it was found that the cytoplasm of erythrocytes can be stained selectively by means of a 0.1% aqueous solution of acid fuchsin. Subsequent experiments with whole mounts of Tubifex rivulorum proved that this very selective and simple stain for hemoglobin can also be used for the selective demonstration of erythrocruorin in invertebrates.**

Up to now there has been only one method that makes possible the selective demonstration in microscopic balsam preparations of both hemoglobin in vertebrates and erythrocruorin (Svedeberg and Eriksson, 1933) in invertebrates, both in the cellular and the dissolved state. This is the method of Lepehne (1919) which depends on the reaction of the red blood pigments with benzidine, a reaction which is also obtained with peroxidases under different experimental conditions. After Prenant (1921) had shown that the method of Lepehne can also be applied to the "hemoglobins" of invertebrates, the method was modified by Slonimski (1927), Slonimski and Lapinski (1932) and Doherty, Suh and Alexander (1938) and its utility thereby considerably enhanced. The method described in this paper has nothing in common with the basic principle and the modifications of Lepehne's reaction, although it is just as selective, and applicable to the same objects.

The method was discovered in the following way: smears of Amphibian testis were fixed in aqueous sulfosalicylic acid solution, run through a series of descending alcohols to distilled water and stained with iron hematoxylin (Heidenhain). A very selective nuclear stain is obtained after this fixation, but since I also wanted to observe the cytoplasm of the cells, the smears were dipped for about 1 minute into a weak aqueous solution of acid fuchsin. After mounting in Canada balsam, it was noticed that only the cytoplasm of erythrocytes was stained bright red while other cells remained completely unstained.

[1]Translated from the German original by Max Alfert, Columbia University.

Control-smears of Amphibian testis, fixed and prepared as before, showed the same selective and intensive staining of erythrocytes, although no mordant and indeed no staining at all preceded the acid fuchsin stain. It became clear that this method constitutes a very selective and simple staining procedure for hemoglobin.

This induced me to determine whether the erythrocruorin of invertebrates could be demonstrated by the same method, and the small aquatic worm *Tubifex rivulorum* was used for this purpose. This worm, which occurs in large numbers in the mud of brooks, contains the red blood pigment not in blood cells, but like other invertebrates (for instance the larvae of *Chironomus*) in colloidal solution in the blood. The experimental animals were first isolated in small glass containers in clean tap water for 4–5 days; during this time their intestine was emptied of mud which would have partially obscured the course of the blood vessels in the whole preparations required for this purpose. Then the animals were anesthetized by means of ether, dried with blotting paper and put on a glass slide. They were covered with a smaller slide that had been dipped into paraffin and weighted with a small piece of lead, also dipped into paraffin. The covering with a slide only slightly larger than the animal allowed easy access to the fixative, and the paraffin covering made this slide easily removable after alcohol hardening. The weighting of the cover slide flattened the animal so as to permit observations with stronger objectives and the paraffin coating of the weight prevented the lead from dissolving in the fixative. It was found that previous drying with blotting paper removes excess water and causes the object to adhere strongly to the slide. Sulfosalicylic acid being a very strong and rapid protein coagulant causes the object to adhere especially strongly to the slide and diffuses very rapidly—like Carnoy's mixture—through the tissues. But unlike the last named fixing fluid it does not permit any nucleal staining (Feulgen). The narcosis of the animals should not be prolonged and everything should be done rapidly to get them into the fixative with as little alteration as possible.

For this purpose the slide bearing the object is lifted by means of two forceps and put carefully—in horizontal position—into an aqueous solution of sulfosalicylic acid kept ready in a wide pan. The following details of the process are indicated:

1) Fix smears and very small animals for 24 hours at 20–25°C. in 5% sulfosalicylic acid in water.

2) Transfer whole animals into 2 successive 24-hour baths of 100% alcohol. Wash smears in 24-hour baths of 100% and 95% alcohol.

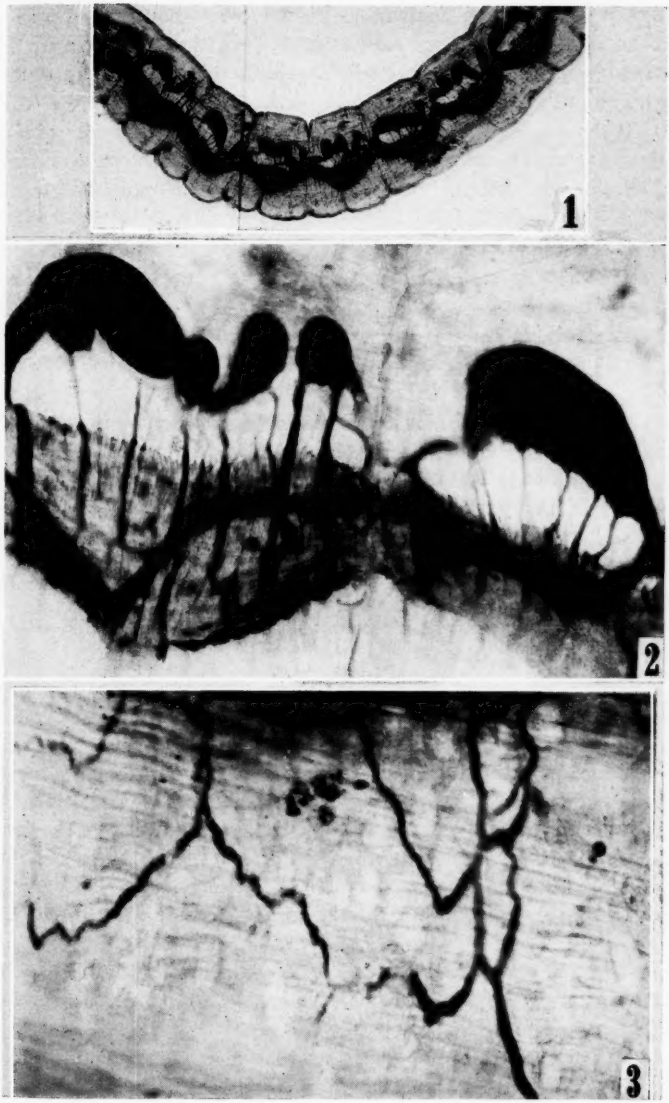

PLATE I. EXPLANATION OF FIGURES

Photographs of whole mounts of *Tubifex rivulorum* stained with acid fuchsin, showing selective staining of blood vessels.

FIG. 1 (× 50) FIG. 2 (× 250) FIG. 3 (× 520)

3) Hydrate whole animals in successive 2- to 3-hour baths, smears in 5- to 10-minute baths, of 90, 80, 70, 60, 50% alcohol. Remove the covering upper slide from the whole objects at the beginning of this step, and put slide vertically into the dish.

4) Wash out alcohol in several changes of distilled water over a period of several hours in the case of whole animals, 1 hour for smears.

5) Stain small animals such as *Tubifex*, up to 20 minutes (smears for 1 minute only), in 0.1% aqueous acid fuchsin.

6) Rinse small animals 2 to 3 seconds (smears 1 second), in distilled water. Transfer to 80% alcohol, agitating constantly to quickly remove water, for at most 10 minutes.

7) Treat whole animals and smears in 90% alcohol for 10 minutes or less. Dehydrate smears 20 minutes, small animals 1 to 2 hours in 95% alcohol.

8) Complete dehydration and clearing with 2 changes of 100% alcohol, a 1:1 mixture of 100% alcohol and xylene, and 2 changes of xylene. Mount in balsam.

Fig. 1, 2, and 3 show how selective this method is; the figures show parts of mounted, whole *Tubifex*.

Fig. 1 (×50) shows the larger vessels dark red (here black) on unstained background in a series of segments.

Fig. 2 (×250) shows smaller vessels selectively stained in 2 segments.

Fig. 3 (×520, under oil immersion) shows fragments of capillary anastomoses. Although the staining of the vessels is caused only by the selective staining of erythrocruorin, the illustrations give the impression of injected preparations. The light-grey appearance of other structures, like the intestine in Fig. 1 and 2 or muscle tissue in Fig. 3, is not caused by staining but by a strong reduction of the light which had the purpose of giving considerable depth to the photographs. There is a sole exception to the selectivity of the method, which however is hardly disturbing: in *Tubifex* certain components of the yolk of old oocytes are, interestingly enough, also stained, while all other organs and tissues remain completely unstained. The durability of the preparations is very satisfactory, almost a full year according to my experience.

This paper is based on experiments undertaken at my former Zoological Institute of the University of Lemberg before 1940 and partly on experiments done at the Zoological Institute of the University of Vienna in 1940. For the opportunity to work there I am indebted to Professor Dr. W. Frh. v. Buddenbrock, the former chief of that Institute.

The photographs shown here were taken by Ing. A. Findeis of the C. Reichert Optical Company in Vienna. I thank him for his friendly cooperation.

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A RIGOROUS RE-DEFINITION OF THE PLASMAL REACTION

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Abstract.—**The plasmal reaction is here modified so that it is made strictly specific for acetal lipids alone. The two essential points in making such specificity possible are: (1) limiting the duration of the action of $HgCl_2$ to 2–10 minutes, thus insuring that neither acid hydrolysis nor oxidation participates in the unmasking; (2) obtaining negative controls upon sections of the same block of tissue, these being treated identically in all respects except that immersion in $HgCl_2$ is omitted. Lipids positive under these conditions are acetals but not necessarily acetal phospholipids. In order to satisfy the second criterion above, material must be either unfixed or fixed for only 1–6 hours in formol. Longer fixation in formol both destroys the acetals and unmasks some non-acetal lipids.**

There has been much discussion concerning the histochemical demonstration of carbonyl lipids and fundamentally different opinions have been expressed as to the chemical significance of the methods used. The principal contrasting points of view of American workers are adequately expressed in two recent papers, those of Albert and Leblond (1946) and Wislocki and Wimsatt (1947). Both agree with Gomori (1942) that the plasmal reaction of Feulgen and Voit (1924) is equivalent to the phenylhydrazine reaction of Bennett (1940). They disagree as to whether the lipids visualized are the plasmalogens (acetal phospholipids) of Feulgen and Bersin (1939) or whether they are to an important extent ketosteroids.

The present writer will publish elsewhere the results of an investigation of the significance of the carbonyl lipid reactions. As a necessary preliminary to the study of whether the plasmal reaction and Bennett's reaction are identical, a more rigorously defined plasmal reaction was devised and used. The specificity of this reaction is strictly limited to acetal lipids alone. This paper presents the details of the method.

Carbonyl reagents do not react directly with untreated sections or smears, meaning that tissues contain no free carbonyl compounds in histochemically detectable amounts. Carbonyl groups must first be freed from some masking linkage before they will react. Two

classic examples of this are the nucleal reaction of Feulgen and Rossenbeck (1924), in which short acid hydrolysis releases carbonyl groups from desoxyribose nucleotides, and the Bauer (1933) reaction, in which oxidation makes glycogen reactive to carbonyl reagents. The ultimate reagent used to visualize the unmasked carbonyl compounds has no influence upon the histochemical picture obtained and thus, presumably, none upon the chemical specificity of the reactions. It follows, then, that the specificity of any reaction for carbonyl lipids derives solely from the method used for the unmasking of the reactive products and does not depend upon whether Schiff's reagent, phenylhydrazine or 2, 4–dinitrophenylhydrazine is used.

There are three methods for the unmasking of carbonyl lipids: (1) brief action of $HgCl_2$, (2) more prolonged acid hydrolysis (or the action of the acidic Schiff reagent itself), and (3) formol fixation. The first two methods are included in the original definition of the plasmal reaction by Feulgen and Voit (1924). The third method derives from the work of Bennett (1940); Gomori (1942) suggests that the mechanism in this case is oxidative.

The striking action of $HgCl_2$ upon the acetal bond, splitting it with the formation of a free carbonyl compound, was fundamental to the discovery of the acetal lipids. This lability of plasmalogen toward $HgCl_2$ enabled Feulgen and Bersin (1939) to recognize that the fatty aldehyde and glycerol of plasmalogen were joined in an acetal linkage. Riesch and Kilpatrick (1935) have described the catalysis of the hydrolysis of the acetal bond by neutral salts, and Meerwein (1927) explains this on the basis of complex formation. Oster and Schlossman (1942) say that $HgCl_2$ acts "more specifically and quicker" than hydrolysis by mineral acids. It is, of course, necessary to establish the lipid nature of the acetals by extraction of sections in organic solvents.

Acid hydrolysis and formol fixation are less likely to have a specific action upon the acetal bond. Hydrolysis by acid undoubtedly breaks acetal bonds to form free carbonyls but it is quite possible that such treatment may release carbonyls from substances other than acetals. Further, since this process takes an appreciable length of time (1–2 hours), opportunity is offered for other unmasking processes to take place. Oxidation of an acetal bond will split it, but with the formation of an acid rather than a free carbonyl group. Therefore, if the unmasking of lipid substances can be restricted to the action of $HgCl_2$ alone, it is certain that the reactive lipid originally contained an acetal bond. We cannot, however, be certain that these were phospholipids, for the possibility remains that nonphospholipid acetals exist.

Insistence upon two points in the procedure affords assurance that the unmasking is so restricted: (1) very brief action of $HgCl_2$, 2–10 minutes in a 1% solution, to minimize the possibility of the slower actions of acid hydrolysis or oxidation; (2) obtaining negative control sections from the same block of tissue, these sections being treated identically except for the immersion in $HgCl_2$. Any material that reacts positively in the control sections cannot be known to be derived from acetal lipids.

The procedure is as follows:

(1) Use unfixed tissues, either frozen sections or smears. Sectioning of such material is aided if blocks are immersed in a dilute gum arabic solution for 5–10 minutes and sectioned in a drop of this solution. Most material can be cut at 15–20μ by this method.

(2) Wash in several changes of distilled water or physiological saline.

(3) Place one section or group of sections in 1% aqueous $HgCl_2$ for 2–10 minutes. It seems necessary to allow only sufficient time for penetration to be complete.

(4) Transfer these sections and a control group simultaneously to separate closed dishes of Schiff's reagent. Sections may be left here as long as the controls remain negative but 5–15 minutes is usually sufficient for a maximal reaction to be secured.

(5) Wash both groups of sections in 3 changes of H_2SO_3, at least 2 minutes in each.

(6) Wash in water; mount in glychrogel (Zwemer, 1933) or in Clarite.

Schiff's reagent or fuchsin-sulfurous acid is preferred as the ultimate demonstrating reagent. Phenylhydrazine (Bennett, 1940) or its 2,4-dinitro derivative (Albert and Leblond, 1946) may be used. With these latter reagents, the preparations are not as distinct, although the results as regards distribution and relative intensity of reacting substances are identical to those with the Schiff reagent. The various ways of preparing the Schiff reagent do not influence the histochemical pictures of the acetal lipids. It is necessary to use a basic fuchsin that is certified for the Feulgen reaction by the Biological Stain Commission, for not all batches of this dye are satisfactory. The best method of preparation is that of deTomasi (1936), using $K_2S_2O_5$. Decolorization is completed with activated charcoal as suggested by Coleman (1938). This results in a colorless solution in which the first hint of returning color can be detected and the solution discarded.

Glass rod section lifters are used to transfer frozen sections. Care must be taken not to allow even traces of Schiff's reagent or $HgCl_2$

to be carried backward in the series, as this can lead to confusion. Scrupulous rinsing of the lifters after each transfer is advised.

Careful washing in H_2SO_3 is essential to remove all traces of excess reagent that might lead to false staining. This wash solution is conveniently made up as needed by mixing 10 ml. of 10% aqueous $NaHSO_3$ and 10 ml. of *N* HCl and diluting to 200 ml. with distilled water.

Sections mounted in glychrogel do not keep. By taking advantage of the fact that the fuchsin-carbonyl lipid compound is practically insoluble in organic solvents, permanent preparations in Clarite may be obtained. The procedure is to float the section upon a slide and to apply successively 50% alcohol, 95% alcohol, absolute alcohol and xylene. This is followed by mounting in Clarite. If a light nuclear counterstain is desired in such preparations, methyl green is quite satisfactory.

Any fixation that neither destroys nor itself unmasks carbonyl lipids could theoretically be used. The only method of fixation known to the author that allows negative control sections to be obtained is brief (1–6 hours) fixation in 10% formol. This makes sectioning much more satisfactory but destroys some of the acetal lipids. Longer fixation in formol will eventually destroy all the acetal lipids as well as unmask positive material in the control sections.

Addendum

Recently it has been brought to the author's attention by Dr. C. P. Leblond and Dr. R. A. Knouff that lipids of the adrenal glands of the mouse and hamster react quickly and directly with the Schiff reagent without any preliminary treatment. Guyon (Comp. Rend. Soc. Biol. 109, 1101–5, 1932) observed that myelin also reacts in this way. Such lipids cannot be proven to be acetals, for the unmasking has not been restricted to the action of $HgCl_2$. The carbonyl lipids observed in these isolated cases may exist *in vivo* either actually free or in some bond extremely labile to the acid of the reagent.

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THE HISTOCHEMICAL DEMONSTRATION OF GLYCOGEN BY SILVER COMPLEXES

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Abstract.—**Several silver complexes and procedures of hydrolysis for the histochemical demonstration of glycogen were studied. Among these were ammoniacal-silver-hydroxide, methenamine silver, piperazine silver, and ammoniacal silver carbonate. It was found that any combination of acid hydrolysis and oxidation is able to activate the carbohydrate so there is no specific agent although the best was chromic acid. The suggestion is made that periodic acid hydrolysis can yield equally good results in shorter time. Ammoniacal silver carbonate was the best of the complexes studied because it stains rapidly, is economical, stable and easy to prepare.**

Direct or indirect (with formol reduction) reactions can be chosen when working with ammoniacal silver carbonate complexes. The following procedures are proposed: a) hydrolysis with 10% chromic acid, 20 to 30 minutes; b) immerse in the following complex: 10% aqueous silver nitrate, 4 ml.; saturated solution of lithium carbonate, to 20 ml.; drop concentrated ammonium hydroxide until almost completely dissolved (leave a light turbidity); complete to 100 ml. with lithium carbonate saturated solution; let stand and filter. Store in a brown glass well-stoppered bottle. The slides can either be exposed 15 minutes at room temperature in this complex and reduced with 2% neutral formol for about 30 seconds, or incubated during 50 to 60 minutes in a 10% solution of said complex at 45–50°C. in the oven followed by a thorough washing with water. Tone with 1:500 gold chloride and finish as usual. Collodion coating and formol alcohol or picro-formol-alcohol-acetic fixation are essential in direct reactions.

So far as we know three methods for the histochemical demonstration of glycogen by silver complexes have been reported (Mitchell and Wislocki, 1944; Gomori, 1946; Arzac, 1947). In all of them two steps are essential: (a) hydrolysis of the polysaccharide; (b) reduction of the silver by the hydrolyzed carbohydrate itself. Except in Gomori's procedure which is a *direct* reaction, the other two need a third step, namely what we may call "development". While two of them (Mitchell and Wislocki, 1944; Arzac, 1947) use potassium perman-

ganate, with bleaching in oxalic acid and $AgNO_3$ in alcoholic solution, Gomori recommends chromic acid as hydrolyzing agent. For the second step a silver oxide complex of ammonia has been advocated by Mitchell and Wislocki (1944), an ammoniacal silver carbonate by Arzac (1947) and a methenamine complex by Gomori (1946). Since the statement has been made that only methenamine complexes are well suited to be used in direct reactions (in the sense used by Lison, 1936) but we have found ammoniacal complexes extremely sensitive to reduction by glycogen, the present study was undertaken to get information on the possibilities of: (a) replacing or interchanging some steps in both types of histochemical reactions; (b) using other complexes than the ones advocated; and (c) replacing other hydrolyzing procedures than the two previously mentioned.

TABLE 1.—EFFECT OF SEVERAL HYDROLYZING PROCEDURES AND SILVER COMPLEXES FOR THE HISTOCHEMICAL DEMONSTRATION OF GLYCOGEN

Hydrolysis ⟶	A $KMnO_4$ Oxalic Acid Alc. $AgNO_3$*		B $KMnO_4$ Oxalic Acid Aq. $AgNO_3$		C $KMnO_4$ Oxalic Acid Acid $AgNO_3$†		D Chromic Acid‡	
Complex—↓	F	H	F	H	F	H	F	H
Metheneamine-Ag	+	+	−	−	+	+	+	+
Ammoniacal-Ag	+	+	−	−	+	+	+	+
Piperazine-Ag	+	+	−	−	+	+	+	+

*2 to 10% silver solutions (24 hours).
†pH 2 with acetic acid.
‡10% sol. 20–30 minutes.
F = Reduction with formol.
H = Incubation at 45–50°C, or 18–24 hours at room temperature.
B and C = Silver solutions in water.

EXPERIMENTAL

Endometrium, liver, stomach and intestine, spleen, etc. from rodents and human, freshly fixed in alcoholic mixtures, were used. Digestion with saliva (as well as fixation in fluids which do not preserve glycogen) was previously performed when necessary. The different combinations of steps are shown schematically in Table 1. In all cases a double procedure was tried: (a) reduction with formol 0.2 to 4%; (b) acceleration by heat (45°C., no formol), or incubation in the silver complexes during 18–24 hours at room temperature.

RESULTS AND COMMENT

It can be seen in Table 1 that in all cases except B (in which the acidity of alcohol or some acid is lacking) a positive result was obtained in every instance. When formol was used the results were essentially the same for all the combinations of steps and reagents

tried; this was also true when heat was applied, the only essential difference with the latter group being the absence of reticulum. For example, glycogen or mucus plus reticulum (or both) was demonstrated in all the experiments on endometrium, when reduction with 0.25–4% formol was used. When the reaction took place at 45°C. over a period of 30 minutes to 3 hours (18–24 hours at room temperature) reticulum did not appear while glycogen or mucus was demonstrated.

The formulas tried for each complex were varied widely in concentration, solvent and alkalizing agent. It was found that lithium carbonate was the best for ammoniacal and piperazine silver, while ammonium hydroxide for Gomori's was to be preferred. Piperazine works well without any alkalizing agent.

Although the silver concentration of the complex and the speed of the reaction run parallel, from a practical point of view high concentrations are to be preferred when formol is used as reductant since the complexes are quite stable and thus may be used repeatedly.

TABLE 2.—RESULTS OBTAINED WITH SEVERAL SILVER COMPLEXES.

	Method	Carbohydrates	Reticulum
Reduction with formol.	Mitchel and Wislocki's	Sharply outlined	Sharply outlined
	Piperazine-silver	Sharply outlined	Not so
	Methenamine-silver	Sharply outlined	Not so
	Ammonium silver-carb.	Sharply outlined	Still less
No reduction	Mitchel and Wislocki's	Blurred	Blurred
	All the others	Well outlined	No reticulum

Glycogen and mucus appear black; grayish purple when scanty and diffuse.

This is particularly true for ammoniacal carbonate complexes. Furthermore silver concentrations as small as 0.004% are sufficient for the demonstration of glycogen in 15 minutes at 45°C. However, sharper images with glycogen and jet black mucus are obtained in 10–15 minutes when 0.25% silver concentrations and formol reduction are used in slides without collodion membrane (see Plate 1). In our experience methenamine silver did not even begin to stain in this length of time at 37–45°C.

Regarding the procedures for hydrolysis, it can be deduced from the results shown in Table 1 that there is no specific hydrolyzing agent for glycogen since appropriate oxidation combined with a more or less independent acid hydrolysis works perfectly. In line with this, Lillie (1948) and McManus (see Wislocki and Dempsey, 1948) have demonstrated that hydrolysis can be performed by periodic acid which fulfills the same requirements, that is acid hydrolysis with

oxidation. Of the methods studied in this work chromic acid hydrolysis was the best. However, it is probable that hydrolysis by periodic acid (not available to us) used with the methods below might give as effective results in shorter time.

In relation to specificity of the technics it can be said definitely that none of the technics so far proposed, either by silver, leucofuchsin or by chromic acid etc., is strictly specific *per se;* all of them demonstrate structures and substances other than glycogen and mucus, so from this particular point of view none of them has advantage over the others. Certain differences, however, can be established from a practical standpoint as shown in Table 2. It can be assumed that progressive reduction of silver by the carbohydrate, while only *adsorption* upon reticulum, takes place in both types of reactions. At high temperatures reduction and blackening of silver by the carbohydrate take place automatically while the latter is carried away from reticulum and other structures with washing. Direct reduction of silver by the carbohydrate is on the contrary too weak when exposed to short periods of incubation or low temperatures so the use of formol is necessary for its visualization. This of course simultaneously reduces and makes visible the silver adsorbed upon the reticulum fibers.

The results of the experiments here reported show, we believe, that because of the many variations that can be made in the ammoniacal silver carbonate complexes (as well as in the technics), the simplicity of their preparation and relatively greater stability and speed for staining, they are preferable. For the investigation of certain tissues like human endometrium, in which variations of reticulum argyrophilia (Fig. 4) during the menstrual cycle represent functional state or activity, an ammoniacal-silver-carbonate complex combined with weak formol reduction is the best choice. The following formula has been demonstrated to be very useful:

"LI2"

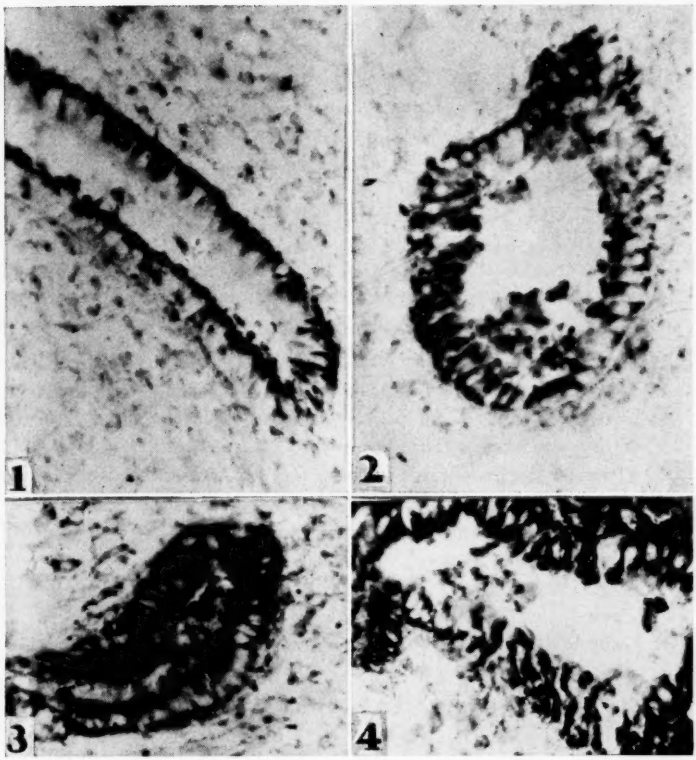
$AgNO_3$ (10% aqueous solution) ..... 4 ml.
Li_2CO_3 (saturated aqueous solution) ..... to 20 ml.

Ammonium hydroxide (NH_4OH) drops, until the complex is almost dissolved (leave a very light turbidity)

Li_2CO_3 (saturated aqueous solution) to make 100 ml.

Filter and keep in a dark well-stoppered bottle in refrigerator.[1] This stock solution can be used repeatedly in full strength (with

[1]After 3 months at room temperature this solution has remained unchanged and active.



Human endometrial glands. All from same specimen E. 942 fixed in alcohol-formol. Effect of several silver complexes after 10% chromic acid hydrolysis during 25 minutes.

FIG. 1.—Methenamine silver. Temperature 50°C. Time: 1 hour

FIG. 2.—Piperazine silver (SEP5b). Temperature 50°C. Time: 30 minutes

FIG. 3.—Ammoniacal silver carbonate (LI5b). Temperature 50°C. Time: 30 minutes.

FIG. 4.—Ammoniacal-silver-carbonate (LI2) Room temperature. Time: 15 min. Reduction with 2% neutral formol. Almost no reticulum appears in late progestational phase.

formol reduction) or diluted 1:10 (we call it LI5b) in the oven (without reduction) once or twice. A piperazine silver complex that has worked very well in our hands is the next one:

"SEP1"

Piperazine solution, 0.5%	100 ml.
$AgNO_3$ (10% aqueous solution)	1 ml.

This is stock solution. For use dilute 1:4 with distilled water. (SEP5b). Two drops of Li_2CO_3 (saturated aqueous solution) to 50 ml. of SEP5b may be added.

We recommend the following technics: deparafinize and run down the slides to water protecting with collodion[2] as usual and then:

1. 10% chromic acid solution ——— 20–30 min.
2. Wash thoroughly in tap water and rinse in distilled water.
3. LI2 15 min. at room temp. or in LI5b (10% aqueous solution) or in SEP5b at 45°C. 15–60 min. (Heat the reagent in the oven while hydrolysis is taking place).
4. After cooling wash in water. (In 2% neutral formol 30–60 seconds and then in water if worked at room temperature).
5. Tone with 1:500 gold chloride if desired.
6. Rinse in 3–5% "hypo", 3–5 min.
7. Wash thoroughly. Take off collodion membrane with alcohol-ether.
8. Dehydrate, clear, and mount as usual.

Summary and Conclusions

Any kind of pretreatment which involves acid hydrolysis and oxidation permits the histochemical demonstration of glycogen and mucus by silver complexes. Of the procedures that we used chromic acid was the best.

A relatively wide variety of silver complexes are well suited for the histochemical demonstration of glycogen and mucus. Ammoniacal silver carbonate is recommended.

None of the technics so far proposed is specific. Reduction with formol widens the range of non-specificity for all of them but gives sharper images and quicker results.

A fixative containing formol and alcohol, and coating of the slides

[2]In our technic previously reported (1947) we used alcoholic silver solutions and complexes to avoid the use of collodion. Although both types of the technic here recommended can be worked using alcoholic complexes, aqueous ones are more satisfactory and smaller concentrations are required.

with collodion are essential in "direct" reactions, although much less important in "developed" ones.

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CONCENTRATED FORMALIN VERSUS A 10% SOLUTION AS A FIXATIVE PRECEDING SILVER STAINING

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Abstract.—**The effect of concentrated and 10% formalin as fixatives for the silver staining of axons in the central nervous system in a series of mammals has been compared. It is felt that concentrated formalin constitutes a better fixative for this purpose in several respects: it has a greater speed of penetration without causing additional distortion or artifacts, the staining time can be reduced somewhat and the normal configuration of the axons is better preserved particularly in the largest ones. Satisfactory results were obtained following 5½ hours fixation in concentrated formalin. Further, concentrated formalin is at least equal to a 10% solution in the ease in which paraffin and frozen blocks can be sectioned, in the clearness of the background of stained sections and in the consistency of results.**

The purpose of the present investigation is to compare the possible value of concentrated formalin (40% commercial formalin, or formaldehyde) with that of a 10% solution as a fixative for the silver staining of axons in the central nervous system.

Since 1893, formalin has largely superseded Müller's fluid as a fixing reagent for the tissues of the brain and spinal cord. It is almost universally accepted that it is best employed as a 10% solution of concentrated commercial formalin. In this percentage, it is widely used singly as a fixative in various axonal staining procedures which employ either silver nitrate or protargol.

Some studies have been made on the histological effects of formalin on the tissue of the central nervous system, notably by King (1910, 1913). She tested only solutions of 10% formalin on the brain of the albino rat and found that it caused considerable general swelling. She concluded that it was not a satisfactory fixative to show the cell structure of the brain, as it causes a pronounced distention of the nuclei with poor preservation of the nuclear contents. On the other hand, she stated it does not adversely affect the nerve tracts as determined by the Weigert stain. She did not employ higher concen-

trations of formalin in her experiments nor did she investigate the effect on the axis cylinders by means of silver staining.

Concentrated formalin solution is a fluid which has a high specific gravity, 1.124; its pH is in the acid range, and it is known to be excellent in respect to tissue penetration.

Some of the factors that are of importance in judging the value of any fixing reagent may be the following:

1. Speed and thoroughness of penetration.
2. Degree and consistency of hardening of fixed tissues.
3. The manner in which the tissues section.
4. The subsequent selectivity and degree of sharp staining it permits of specific as well as background elements.

It is not the intent of this investigation to determine the merit of concentrated formalin as a general fixative but merely to compare it with the commonly used 10% formalin in respect to the factors listed in the preceding paragraph.

Material and Methods

About 1,500 sections from 635 paraffin-embedded blocks of nervous tissue have been made from the spinal cords and brain-stems of 18 animals: 2 rabbits, 2 rats, 11 cats, 2 monkeys and 1 human. The blocks varied in length from about 3 to 5 mm.

The times alloted for fixation in non-neutralized concentrated formalin at room temperatures (average, 24°C., 75°F.) were the following: 15, 30, 45 and 60 minutes; 2, 3, 5½, 7, 10½, 12 and 24 hours; 3, 4, 5, 6, and 7 days and then removal at weekly intervals up to 2½ months. In all the experiments, immersion of blocks for the same durations in 10% formalin were used as controls.

In the majority of instances, fixed tissues were washed overnight in running water, then run up through the alcohols and xylene and eventually embedded in 60–62°C. rubber paraffin. In a limited number of blocks from each animal, dioxane (for 24 hours) was tried as a dehydrating agent.

For the staining procedures, Bodian's protargol method (1937), Davenport's silver nitrate method including gold toning (1930) and the 2-hour staining method with protargol of Davenport *et al* (1939) were used. Except for the minor variations listed below, the above technics as originally presented have been followed.

Davenport's silver nitrate method (1930) for paraffin sections consists basically of impregnating with an alcoholic silver nitrate solution and then reducing in a mixture of pyrogallol, formalin and ethyl alcohol. After reduction by the above method, we placed

slides in a 5% solution of sodium thiosulfate for 5 minutes to eliminate any possible residual salts.

In the technic for the staining of axons with protargol developed by Davenport et al (1939), we obtained better results by cutting down the time from 1 hour to 20 minutes while the sections were being impregnated in both 10% aqueous silver nitrate and 0.2% protargol solutions.

Results

Grossly, a difference could be detected between the fixing reaction of concentrated and 10% formalin on tissue of the central nervous

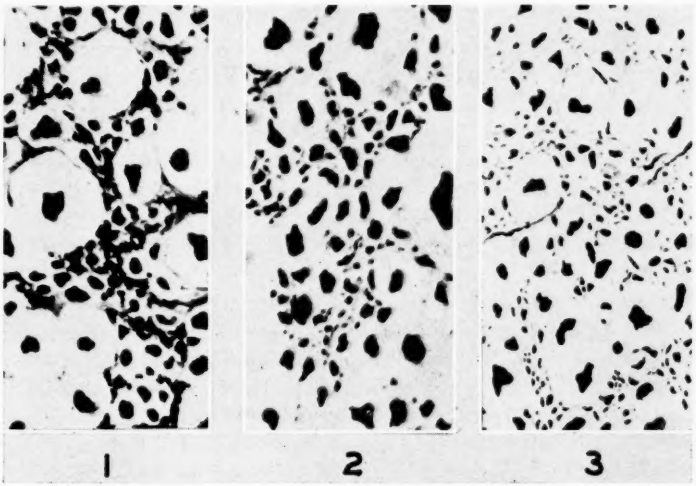

Fig. 1 and 2 show the silver staining response of axons in the anterior funiculus of the spinal cord of the cat following 5½ hours and 5 weeks fixation in non-neutralized formaldehyde (40% commercial formalin) respectively. Staining: Davenport's silver nitrate method followed by gold toning.

Fig. 3 shows the axonal reaction in the anterior funiculus of the spinal cord of the rabbit following 5½ hours fixation in formaldehyde. Staining: Bodian's activated protargol method.

system. Because of the high specific gravity of the former, the blocks float or remain near the top surface of the fluid at first, but within 2 to 3 hours they gradually fall to the bottom. The thoroughness of penetration can, to some extent, be correlated with the sinking of the blocks. The same is not true for 10% formalin as the specimens sink immediately upon immersion. The 10% solution

causes the cut ends to become very fuzzy for a week or so. The material which accumulates on the cut ends then drops off and forms a sediment in the fluid. In contrast, the specimens fixed in concentrated formalin retain an almost glassy smoothness throughout the duration of submersion. It may be that this is the key to an understanding of the different results we have obtained between the two mediums under consideration.

The penetrating power of concentrated formalin is much more rapid than that of 10% formalin. Small axons will stain throughout the cord after only 15 minutes fixation in concentrated formalin. After 5½ hours fixation in the same fluid, both large and small axons are well differentiated (Fig. 1 and 3), and this time is considered to be near the minimum necessary to harden the specimens adequately although very satisfactory preparations were often obtained after 3 hours fixation. Prolonged submersion, up to 10 weeks in concentrated formalin, does not seem to alter the staining response.

In all of the blocks of cord and brain-stem which were sectioned, it was found that those fixed in concentrated formalin cut just as easily and satisfactorily as those subjected to the action of 10% formalin. No difficulty was encountered at any time in respect to ribboning, flattening of the sections, or in tearing and distortion of the tissues.

The main advantage of concentrated formalin-fixed tissues, as far as staining is concerned, appears to be that it is followed by much less shrinkage of the axis cylinders and particularly of the largest ones. Much better results were obtained with Davenport's silver nitrate method (1930) and with Bodian's technic (1937).

The cells also seem to undergo less shrinkage and distortion following fixation in concentrated formalin. Although no direct effort was made to stain any of the peripheral nerves, it was noticed that the dorsal and ventral roots responded well and perhaps better following fixation in concentrated formalin.

Dioxane, as a dehydrating agent, works satisfactorily following fixation in concentrated formalin but the axons appear to have slightly less affinity for the silver stains.

A number of attempts were made to test the ease of cutting tissues fixed in concentrated formalin with the freezing microtome. Tissues which were fixed from 2 to 24 hours and then frozen, sectioned, as nearly as we could judge, about the same as those subjected to 10% formalin.

Comments

On the basis of the results obtained, it seems that concentrated formalin constitutes a reagent which may serve as a better fixing

medium than the diluted 10% formalin for the staining of axis cylinders in the central nervous system of mammals. We believe that it is superior in several respects. It has a much more rapid penetrating power without causing any additional distortion; it affects the tissues so that the staining time can be reduced somewhat and it allows better preservation of the normal configuration of the axons and particularly of the largest ones. It is equal to, or may excel, 10% formalin in the following: in the ease of sectioning paraffin and frozen blocks, in the clearness of the background picture in the staining procedure, and in the consistency of results.

We are not in a position to say what the phenomenon may be which occurs on the cut surface of cords placed in 10% formalin. The characteristic fuzzy appearance, which is not seen in specimens fixed in concentrated formalin, may be due to swelling of the myelin or to some other factor or factors. Whatever the accumulated material at the cut surfaces may be, it is eventually liberated in a week or two to form a sediment in the fluid medium.

It is not claimed that concentrated formalin constitutes a perfect fixative for the staining of axons with silver compounds. However, in our hands, it has been judged to be consistently superior to 10% formalin and our results have been such that further experiments are in progress.

Conclusions

In a series of experiments on the brain-stem and spinal cord of the rat, rabbit, cat, monkey and man, concentrated seems to excel 10% formalin as a fixative for the staining of axons with silver nitrate or protargol compounds. It has proved to be better in respect to speed of penetration without causing additional distortion, in allowing a slight reduction in the staining time and in permitting better preservation of the normal configuration of the axons and particularly of the largest ones. Further, it is at least equal to 10% formalin in the ease in which paraffin and frozen blocks can be sectioned, in the clearness of the background of stained sections and in the consistency of results.

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BRILLIANT CRESYL BLUE AS A STAIN FOR PLANT CHROMOSOMES

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ABSTRACT.—**Methods are proposed for staining plant chromosomes with the dye brilliant cresyl blue, and for making these stained preparations permanent by using polyvinyl alcohol mounting medium.**

The stain, which is composed of 2% brilliant cresyl blue in 45% aqueous acetic or propionic acid, is used with fixed material in making smear preparations. The technics for staining are similar to those employed in the aceto-carmine method.

The mounting medium is made by mixing 56% polyvinyl alcohol, which is diluted in water to the consistency of thick molasses, with 22% lactic acid and 22% phenol by volume. The permanent slides are made by floating off the cover slip of the temporary slide in 70% alcohol, then applying the mounting medium and replacing the cover slip.

The chief advantages of the methods described are:

1) **The preparation of the stain is rapid and simple. The batch of stain will be good with the first try.**
2) **The staining procedure in some instances is shorter than when using aceto-carmine.**
3) **The stain shows a high degree of specificity for nuclear structures and gives better results than aceto-carmine when used on certain plant tissues.**
4) **A minimum number of cells is lost in making the slides permanent when using polyvinyl alcohol mounting medium as the slide and cover slip are run through only one solution prior to mounting.**
5) **The mounting medium dries rapidly and this shortens the time required before critical examination of the permanent mounts can be made.**

INTRODUCTION

In attempting to make a quick determination of the number of divisions occurring at a given time in the root-tip cells of *Phaseolus aureus*,[1] the standard aceto-carmine method was tried with poor

[1] *P. aureus* is being used as an experimental plant by Dr. H. Quastler, Mrs. R. Baer, and the authors to test radiation effects.

results. A modification of the brilliant cresyl blue method, used as a stain for the salivary-gland chromosomes of *Drosophila macrospina macrospina* (Mittler and Bartha, 1948) was then applied to *P. aureus*, and subsequently to the root tips of *Allium*, *Zea*, and *P. vulgaris*, and the anthers of *Rhoeo*.

The stain. Brilliant cresyl blue is a basic dye which has served in the past as a vital stain and as a stain to bring out reticulated blood corpuscles (Conn, 1940). The preparation of this stain, as used in the following technics, is a simple procedure. Two per cent brilliant cresyl blue[2] is dissolved in 50% aqueous propionic acid and filtered. Equally good results are obtained with 45% glacial acetic acid in place of the propionic acid. It is not necessary to boil and decant as in the preparation of aceto-carmine (Smith, 1947), nor is it necessary to add iron. It has been found that this acid solution of the stain brings about distinctly more selective staining of the nuclei than either plain aqueous or plain alcoholic solutions. There is little chance that the stain will not be satisfactory with the first attempt.

The stain, prepared in the manner described above, has an ink-like density. However, this does not prevent microscopic examination of the cells on the slide during the staining process. It is possible to see the cells clearly at the periphery of the drop of stain prior to the application of the cover slip.

The chromatin of the interphase nuclei shows a striking affinity for the stain in contrast to the nucleoli which do not stain at all (Fig. 2, 4). Chromosomes may retain a degree of translucency after staining which reveals their structure at various stages of division. These nuclear structures are stained red-brown to nearly black, while the cytoplasm remains colorless or nearly so, depending on the plant tissues used (Fig. 1–8). In temporary slides the colorless cells with their dark-staining nuclei appear in sharp contrast against the blue background.

With illumination from an ordinary microscope lamp a white diffusing filter is desirable plus a blue filter if the stain is light.

Fixation. All the plant tissues tried stained in 2% brilliant cresyl blue alone, but the resulting fixation was poor. The best results were obtained by staining material which had been fixed in 3 parts of 95% ethyl alcohol and 1 part of glacial acetic acid (Farmer's fluid), or the above modified to contain 2 parts of 95% alcohol and 1 part of glacial acetic acid.

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[2]The lots used are old and bear no certification numbers. They are products of the Coleman and Bell Co., Norwood, Ohio.

Root-tip Smears

The technic used in making root-tip smears of *Allium* served as a basis for making similar preparations of *Phaseolus* and *Zea*.

After fixation, the root-tip smears of *Allium* are made as follows:

1) Place the root tip in a vial or preparation dish containing 1 part of concentrated hydrochloric acid and 1 part of 95% ethyl alcohol. Maceration should be complete at the end of 5 minutes.
2) Transfer the root tip to Farmer's fluid for a period of 2 to 3 minutes.
3) Place in 70% ethyl alcohol for 2 to 3 minutes.
4) On the surface of a clean slide which has been coated with a thin film of Haupt's adhesive place a small drop of stain. Remove the root tip from the 70% alcohol and with a scalpel or razor blade cut cross-sections from the terminal millimeter of the embryonic region. Push these into the stain.
5) Allow the staining process to go on for 3 to 5 minutes, using the scalpel or razor blade during this interval to separate the cells further. At the end of this time examine the cells which accumulate at the margin of the drop of stain to determine the degree of staining.
6) Apply a clean cover slip and remove the excess stain from its margins with a paper towel or blotter. Cover the slide with the paper towel and apply direct thumb pressure on the area of the cover slip, being careful not to let the cover slip move.
7) Seal the slide with a mixture of equal parts of gum-mastic and hard paraffin.

The staining time for *Zea* and *Phaseolus* must be increased to about 10 minutes. The interphase nuclei of *Phaseolus* (Fig. 6) do not take as deep a stain as those of *Zea* and *Allium* (Fig. 2), but the mitotic figures stain deeply (Fig. 5, 6) and are in sharp contrast to the light background.

Smear Preparations for Meiosis

The anthers of *Rhoeo*, which had been fixed in Farmer's fluid, were used in the technic which follows:

1) Place the flower on a clean slide, the surface of which has been coated with a thin film of Haupt's adhesive. Add a drop of Farmer's fluid to prevent the flower from drying and dissect out one or more of the anthers over a dark background.
2) Remove the excess fixative with a blotter or towel and add a small drop of 2% brilliant cresyl blue.

Plate 1

FIG. 1.—Anaphase in a root-tip cell of *Allium*. Note the light staining cytoplasm. ×1300.

FIG. 2.—Root-tip smear of *Allium*. This preparation was made permanent by the polyvinyl alcohol method. ×750.

FIG. 3.—Late prophase during the first division of the pollen-mother-cells of *Rhoeo*. The darker background is due to the presence of the stain in the temporary slide. ×1300.

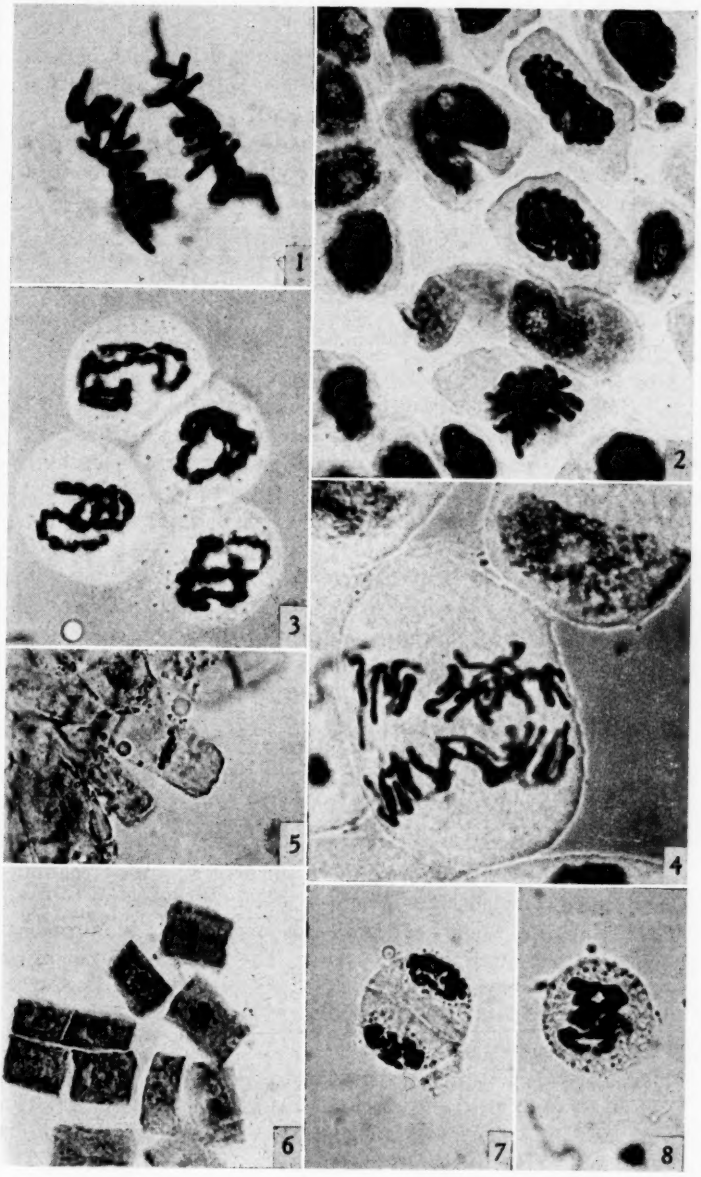
FIG. 4.—Temporary preparation of a root-tip smear of *Allium*. Note the lack of stain in the nucleolus and cytoplasm as contrasted to the dark stain of the chromatin and anaphase chromosomes. ×1300.

FIG. 5.—Metaphase in a root-tip cell of *Phaseolus aureus*. This slide was made permanent by the polyvinyl alcohol method. It was a month old at the time the photograph was taken and there was no sign of fading. ×750.

FIG. 6.—Prophase and metaphase in a root-tip cell of *Phaseolus aureus*. This preparation was made permanent by Bradley's modification of Bridges' alcohol vapor method. Note that the cytoplasm is darker than in those slides made permanent by the polyvinyl alcohol method. ×750.

FIG. 7.—Second division prophase in a pollen-mother-cell of *Rhoeo*. This slide was made permanent by the polyvinyl alcohol method. ×750.

FIG. 8.—First division anaphase in a pollen-mother-cell of *Rhoeo*. The treatment is the same as described above. ×750.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3) Break open the anthers in the stain and with a small scapel squeeze the pollen-mother-cells out.
4) After 4 minutes of staining, examine the cells which have accumulated at the periphery of the drop of stain. If the stain is not dark enough, allow a longer time.
5) If the stain is dark enough, remove the debris and put a clean cover slip in place. Cover with a paper towel and apply direct, gentle thumb pressure.
6) Seal with the gum-mastic-hard-paraffin mixture.

The structure of the meiotic chromosomes of *Rhoeo*, stained with brilliant cresyl blue, is well defined (Fig. 3, 7, 8).

Making Permanent Slides

After the first temporary slides made were about a week old, it was noted that the stain had started to precipitate. In addition, the stain faded from the *Phaseolus* and *Zea* preparations. These two difficulties were overcome by making the slides permanent shortly after the cover slip was first applied.

Of the technics tried for making permanent slides, two were found to be more successful than the others. The first of these is the modification of Bridge's alcohol vapor method as reported by Bradley (1948), which was selected as a technic to eliminate loss of cells. The second technic involves the use of a plastic mounting medium according to the formula given by Huber and Caplin (1947). They give the following instructions for its preparation:

1) 15 g. of polyvinyl alcohol ("Elvanol" 71–24)[3] is added slowly to 100 ml. of cold water.
2) Stir and heat in a water bath to 80°C. until it has the consistency of thick molasses.
3) Filter if necessary with a cheesecloth.

The complete mounting medium consists of 56% of the stock solution to which is added 22% lactic acid and 22% phenol by volume. This mixture dries rapidly and there is a tough seal at the periphery of the cover slip after 12 to 18 hours. The excess dried mounting medium is removed with water.

After staining with brilliant cresyl blue as previously outlined, the mounting medium is applied as follows:

1) The slide is supported in an inverted position by glass rods in a Petri dish (staining dishes, etc., will work as well). The Petri dish is filled with 70% ethyl alcohol so that the slide is

[3]This grade of polyvinyl alcohol may be obtained from E. I. du Pont de Nemours Co., Electrochemicals Department, 7 S. Dearborn Street, Chicago, 3, Illinois.

immersed and the cover slip is allowed to float off to the bottom of the dish.

2) Remove the excess 70% alcohol from the slide and cover slip by touching their edges to a paper towel.
3) Apply a very small drop of polyvinyl alcohol mounting medium and replace the cover slip in exactly its former position.

Discussion

The methods described above for staining plant chromosomes with brilliant cresyl blue and for making slides permanent with polyvinyl alcohol mounting medium are of value in that the total time required is shorter than when using other technics.

The preparation of the stain takes only a matter of minutes. The time required for making the temporary smear preparations is about the same as for the aceto-carmine technic. The chief advantage in using brilliant cresyl blue as a stain is that in certain plant tissues it gives better results than aceto-carmine. For this reason it is thought that the brilliant cresyl blue technic may serve as an added tool for cytological use.

After staining with brilliant cresyl blue, the polyvinyl alcohol method for making slides permanent has two advantages. First, the loss of material from the slide and cover slip is minimized. Second, the time required is shortened as the mounting medium dries rapidly and the slides are ready for critical observation after 12 hours.

Acknowledgment

The authors wish to thank Mr. Alexander Bartha for suggesting the use of this dye on plant materials.

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TREATMENT OF *TRILLIUM ERECTUM* PRIOR TO AND DURING MASS PRODUCTION OF PERMANENT SMEAR PREPARATIONS

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Abstract.—**Technics used in handling dormant plants of *Trillium erectum* L. prior to and during microsporogenesis are described. Normal meiosis occurs during storage of the plants at 4–6°C. Approximate times in days required for various stages of meiosis are presented. Schedules used for the mass production of aceto- or propiono-carmine smears of microsporocytes, microspores and pollen tubes are also given.**

Introduction

As material for cytological research and for teaching purposes *Trillium* offers a number of attributes not present in certain other plant species now widely used for these purposes. The present report is presented in response to a number of requests for information on the handling of *Trillium* in the hope that greater use may be made of such an excellent subject for cytological investigations.

The most important factors which make *Trillium* so desirable are (1) a low number (n = 5) of very large chromosomes (up to 20μ in length), (2) the chromosomes can be distinguished morphologically at metaphase and anaphase (Fig. 1, 4), (3) spiral structure can be readily demonstrated without the use of special technics (see Fig. 1, 2), (4) the anthers are large (4–15 mm. in length) and hence yield a large number of microsporocytes (up to 25,000 for each anther), (5) the plants can be obtained readily from commercial sources[1] and (6) a greenhouse is not required.

General Information

The methods given below have been found suitable for *Trillium erectum* L. (ill-scented or purple-flowered wake-robin). The cytological methods are generally adaptable to other species[2] but probably

[1]The Gardens of the Blue Ridge, Ashford, N. C. can supply several species in large numbers.

[2]The taxonomic literature on various Trillium species is reviewed by Barksdale (1938). A few references to cytological investigations are also given.

not the handling of the rhizomes since a number of species (e.g. *T. grandiflorum*, *T. luteum* and *T. undulatum*) complete their meiosis in the fall and have a very long postmeiotic resting stage. Microspore mitosis in such species would be expected to occur in the early spring.

For those who wish to collect their own material[3], it is suggested that they do some preliminary field work in the spring, when the plants are in bloom, to locate and mark an area which contains a sufficient number of plants. This is advisable because the plants are much more difficult to locate during the fall season. Colored stakes or tags can be used to mark different species where more than one species is present. In the northeastern United States and adjacent provinces in Canada, the rhizomes should be dug in late September or October. This date is also a suitable time to order from dealers if material is not available locally. The delivery date can be specified to insure material at the desired time. If plants are purchased, it is advisable to specify large size rhizomes or at least to specify plants containing undamaged flower buds (small rhizomes usually do not contain flower buds).

Mature plants of *Trillium erectum* L. removed from the ground from late September to November usually contain well developed flower buds. The anthers contain microsporocytes in the premeiotic resting or early meiotic prophase stages. When the plants are kept at a temperature slightly above freezing, meiosis will usually take place in two to four months. Normal meiosis apparently will not occur without exposure of the plant to low temperature. In practice, 4–6° C. has been found to be the most satisfactory for storage of the dormant plants. The onset of meiosis can be controlled to some extent by altering the time at which plants are initially placed in cold storage. Plants placed in refrigeration in October will reach meiosis earlier than those put in during November. Where a large number of plants are needed, this is a useful method of prolonging the season when meiotic stages are available.

For cold storage purposes, it is not necessary to plant the *Trilliums* in soil. They will do much better and be more readily accessible if they are simply placed in a glass or other covered container with some moist sawdust, peat moss, vermiculite, etc. to reduce loss of moisture.

If root tips are desired, the rhizomes may be planted in flats in the greenhouse and dug up when tips are needed, or they may be placed in a moist chamber at room temperature. *Trillium erectum* has been

[3]It may be advisable in some cases to obtain a permit from the appropriate authorities before collecting begins.

found to produce fewer new roots under cultivation than certain other species. *Trillium grandiflorum* (Michx) Salisb. for instance, will usually produce a fair supply of root tips in four to eight weeks. Growth of root tips from seeds is a slow process owing to the peculiarly slow and uncertain manner in which *Trillium* seeds germinate (Barton 1944). For best results it is advisable to plant the seeds immediately after removal from the plant so that they have no opportunity to become thoroughly dried.

Material for Biochemical Analysis

If desired, anther contents may be squeezed out and used for biochemical analysis. Two to ten milligrams of material can be obtained from a single set of anthers. The amount obtained varies from plant to plant and increases considerably in older anthers. A 2-mg. sample, for example, is sufficient for quantitative determination of nucleic acids. (Steele, Sparrow and Ottolenghi, unpublished).

Cytological Methods

The stages of meiosis are generally well synchronized in the anthers from a single flower bud. There is, however, some variability, especially where stages of short duration are present and there is frequently a detectable gradient from end to end in an anther. These differences are usually small and often barely noticeable but occasionally there is a poor synchronization of stages, both within and between anthers. When more than one bud is present there is usually, but not always, a difference in stage of development. This frequently proves very useful in obtaining a variety of stages.

As a preliminary to smearing, it is often desirable to make a test slide to determine the stage of meiosis. To do this, a longitudinal slit can be made in the side of the bud (Text Fig. 1) and a part of an anther removed and smeared. If the stage is too early, the plant can be replaced in the cold room and checked periodically until the desired stage is reached. At low temperatures, such plants will usually survive for weeks or months even though the incision has been made deep enough to expose the anthers. However, the open ends of cut anthers act as point of entry for bacterial infection or fungal attack and a small proportion of plants is lost.

A convenient method of labelling the rhizomes is to use small plastic plant labels attached with a fine copper wire. String and paper labels are not recommended as they are frequently destroyed by fungi in the moist containers.

The approximate time required for *Trillium erectum* to pass through various stages of microsporogenesis at 4–6° C. is given in Table 1. The extended duration of certain stages is a great advantage where it is desirable to have material available at a particular stage of division, for experimental purposes. In plants which undergo mei-

osis at normal summer (or greenhouse) temperatures, the much shorter duration of most stages makes them more difficult or impossible to use for certain experimental purposes for which *T. erectum* is well suited.

The times given in Table 1 are only approximate times. It should be clearly understood that the deviation from average is high in many cases. This is particularly true if plants have been given an experimental treatment such as exposure to x-rays which tend to inhibit division for a time. It should also be noted the times given are only for low temperatures (around 4–6°C.). Once late meiotic prophase has been reached, later stages can be speeded up considerably by placing the plants at higher temperatures. This holds also for stages of microspore mitosis, but it tends to make the cell walls thicker and more opaque and the chromosomes less condensed at metaphase.

TABLE 1.—APPROXIMATE TIME IN DAYS REQUIRED FOR VARIOUS STAGES OF MICROSPOROGENESIS IN *Trillium erectum* AT 4–6° C.

Stages	Duration in days
Leptotene-zygotene	>30
Pachytene	18–24
Diplotene-diakinesis	12–16
First division	5–8
Second division	5–10
Post-meiotic interphase	28–40
Microspore first division	8–14

For certain experimental purposes, *T. erectum* has the advantage that different anthers from the same bud may be removed days or weeks apart and thus cells may be obtained at a number of stages, e.g., at both meiotic and microspore divisions, or at both first and second meiotic division. Control material can likewise be provided by removing a single anther or by removing a whole bud where two or more are present. In the latter case, it is recommended that a portion of the rhizome be left attached to each bud to provide food reserves. Single anthers removed whole at late meiotic prophase will undergo first and second meiotic divisions if handled carefully to avoid crushing and are placed in a moist atmosphere or vial. Whole anthers or microsporocytes suspended in White's nutrient have been successfully cultured by Stern (unpublished). Christensen and Ottolenghi (unpublished) have also grown excised anthers in White's nutrient and found that meiosis would proceed from diplotene to the microspore resting stage.

Fixation

For ordinary aceto- or propiono-carmine smears, no prefixation is necessary but 3:1 or 2:1 alcohol-acetic may be used if it is not desired to show internal (spiral) structure. Fixation of smears on slides or cover glasses may vary from less than one minute to many hours or even days. If desired, such material may be stored in alcohol, preferably in a refrigerator for a period of weeks or months. Fixation of whole anthers or large sections of anthers is not recommended.

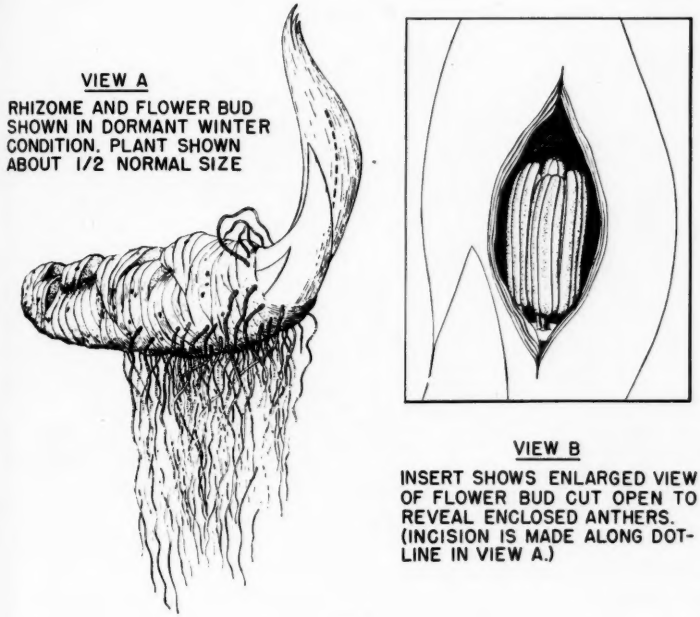
VIEW A
RHIZOME AND FLOWER BUD SHOWN IN DORMANT WINTER CONDITION. PLANT SHOWN ABOUT 1/2 NORMAL SIZE
VIEW B
INSERT SHOWS ENLARGED VIEW OF FLOWER BUD CUT OPEN TO REVEAL ENCLOSED ANTHERS. (INCISION IS MADE ALONG DOT-LINE IN VIEW A.)

Fig. 1.

Schedule for Meiotic Divisions

For the rapid production of a large number of slides, the aceto- or propiono-carmine smear technic (see review by Smith) is preferred by the authors. Special procedures for demonstrating spiral structure have been described by Huskins and Smith, Matsuura, Coleman and Hillary and Sparrow (additional references may be obtained from Kaufmann's recent review).

The following schedule has provided satisfactory material for

routine use and has given a fair number of slides showing clear spiral structure (Pl. 1, Fig. 1 and 2):

1. Clean slides are smeared with a small drop of albumin or Haupt's fixative and heated over a low flame. (Many of these can be prepared at one time and stored in staining jars until needed. Cover glasses are also cleaned in advance.)

2. An anther or half anther is removed from the bud and the contents smeared on an albuminized slide. A drop of iron-aceto or propiono-carmine (McCallum's) is added and the the cells are gently teased with a fine stainless steel dissecting needle (to prevent over-staining due to added iron). The slide is heated slightly over an alcohol flame, the cover glass added and the slide gently pressed under bibulous paper to remove excess stain. Additional heat and pressing may be applied if a quick examination indicates understaining or insufficient flattening.

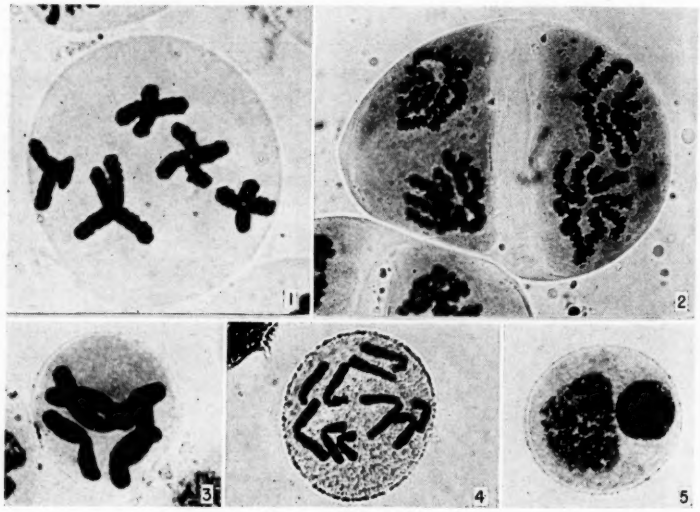

PLATE I

Various stages of microsporogenesis of *Trillium erectum*. Magnification approximately 650×. Stained with propiono-carmine. Fig. 1. First meiotic metaphase (with unusually low number of chiasmata). Fig. 2. Second anaphase showing spiralled chromonemata. Fig. 3 and 4. Microspore metaphase and anaphase respectively. Fig. 5. Binucleate pollen grain showing differentiated nuclei.

3. The covers are temporarily sealed with a mixture of paraffin and gum mastic (1:1). [Slides may be kept for several hours longer without sealing if a small amount (3–6%) of glycerol is added to the carmine staining solution].

4. If well flattened cells are desired, a small lead weight (50–100 g.) can be placed on the cover glass.

5. The slides are stored in a cold room or refrigerator for two to four days before being made permanent. The lead weights can be left in place during this period if the slides are handled carefully.

6. If there are only a few slides to be made permanent, they are handled in flat staining dishes or square Petri dishes as follows (modified from McClintock 1929):

(a) The temporary seal is removed from three sides of the cover glass, but left on the fourth to hold the cover glass in place. Two slides at a time may be placed in 3:1 alcohol-acetic, cover glass side up, for 10–15 minutes.

(b) If necessary, the cover slips are pried loose gently from the free end, but not removed, in order to insure that the alcohol-acetic mixture reaches all the cells.

(c) The slides are removed, drained quickly and transferred to 9:1 alcohol-acetic. They remain in this solution for not less than 5 minutes, when the cover glass is again loosened gently.

(d) The slides are then taken through two changes of absolute or 95% alcohol, remaining in each for at least 2 minutes.

(e) A drop of diaphane or euparol is added and the cover replaced.

(f) Excess diaphane is blotted off with bibulous paper and a small lead weight (50–100 g.) is placed on the cover glass to squeeze out excess mounting fluid.

(g) The weights can be removed after about 2 days but the slides are left flat for about two weeks to dry (less time will be required in a drying oven).

7. When a large number of slides are to be handled, two or three people can work effectively as follows: One person (A) albuminizes slides, cleans cover glasses and runs the previously made slides through the series to make them permanent. A second person (B) smears the anthers on the slides and puts them in 3:1. A third person (C), or B when not otherwise occupied, does the staining, pressing and sealing. If there are a large number of slides to be made permanent, they may be run through the above series in lots of ten or more by using a larger staining dish with removable slide rack. (See also p. 54). In this case, the seal is left along one edge of the cover glass (the edge that will be lowermost after the slide has been placed in the rack). When the first rack of slides is placed in the 9:1, a second set in another rack can be put in the 3:1, and when the second set of slides go into 9:1, a third set can be started in 3:1, etc. This procedure saves a considerable amount of time where many slides are to be processed. An experienced technician should handle 100 slides or more per day.

Schedule for Microspore Divisions

Cells in microspore divisions are handled, in general, in the same manner as meiotic cells. The anthers are smeared on previously albuminized slides and may either be stained immediately, or after prefixation in 3:1 alcohol-acetic. Prefixation of 5–10 minutes is sufficient but slides may be left in the fixative as long as 48 hours. Since the microspores tend to stick together in clumps, especially after prefixation, considerable teasing may be necessary after addition of the stain. Microspores are not as easily damaged as meiotic cells and, therefore, may be handled more roughly. After the clumps of cells are broken up as much as possible with a dissecting needle, the cells may be distributed even more by gently sliding the cover glass back and forth (providing there is a slight excess of stain so that the cover glass is floating).

The slide is then heated and pressed, while still hot, under bibulous paper. A small piece of pressed sheet cork, cut the same shape but smaller than the cover glass, is placed on the cover glass and a heavy lead weight (200–300 g.) is placed on the cork. If convenient, the slides may be left under this weight for as much as 30 minutes before sealing or longer if the stain contains glycerol. This aids in flattening the cells. However, care must be taken that too much of the stain does not evaporate.

If more flattening of the cells is desired, this may be achieved by the following method: Heat the slide over a low burner until quite hot (but not hot enough to boil the stain), quickly replace the cork and lead weight and apply firm pressure with thumb on top of the lead weight. Any excess stain should be wiped off. Then seal the cover to the slide with the paraffin-gum mastic mixture and maintain the pressure until the sealing compound has hardened. The slides are stored in the cold room under the lead weights for 4 days or more before being made permanent (a small amount of glycerol in the stain will reduce evaporation and consequent trouble with air bubbles under the cover glass). Fewer cells will be lost than if the slides are made permanent immediately and the intensity of the stain will usually increase during this time.

A method for the rapid production of a large number of similar slides for class use has been developed:

(1) Lay out 10–20 clean albuminized slides on the table.

(2) Press out the contents of one or more anthers and stain in a very large drop of carmine.

(3) Draw up the suspension of stained cells into a pipette with a capillary tip.

(4) Distribute a small amount of stain and cells to each slide and quickly add a cover glass.

(5) Add more stain at the edges of the cover glass, if necessary.

The slides may be sealed for temporary use or made permanent in the usual manner. Using this technic, as many as 20 slides can be prepared in less than ten minutes.

The method is especially useful for preparing slides for a large class where it is desirable to obtain many slides as similar as possible with the least amount of effort. It is also useful for getting the largest number of slides from a limited amount of material. When a mixture of stages is desired, the contents of several anthers, each at a different stage, may be smeared together, thoroughly mixed and then transferred by pipette to as many slides as possible.

Schedule for Pollen Tube Divisions

Pollen tubes may readily be obtained by germinating pollen on artificial culture media. Fresh pollen is available for only a brief period under natural conditions but some control of blooming time is possible by varying the length of the time the dormant plants are kept in cold storage. Also, mature pollen may be stored for several weeks under controlled relative humidity (around 50%) and still germinate well enough to give a reasonable proportion of pollen tube division figures.

Many methods of growing pollen tubes have been described, and several of these are satisfactory for growing *Trillium* pollen tubes for cytological study. Bishop's (1948) lactose-agar with or without added colchicine has given excellent results. A warm 1 to 3% agar plus 12% (or less) lactose solution containing 0.01% colchicine is spread in a thin layer on a cover glass and allowed to cool. Pollen is dusted onto the agar medium, and the cover glass is then inverted over a depression slide or ring. Humidity can be controlled by a suitable concentration of H_2SO_4 or saturated by a few drops of water placed in the bottom of the chamber. Metaphase figures should be present after 25–50 hours at normal room temperatures. Later stages will also be present if colchicine is not included in the culture medium.

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A SINGLE SOLUTION IRON-HEMATOXYLIN STAIN FOR INTESTINAL PROTOZOA

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ABSTRACT.—**A single solution iron-hematoxylin stain is described for staining fecal smears rapidly and simply. The stain is prepared from the following solutions: Solution A: 1% hematoxylin in 95% alcohol, prepared by diluting a stock solution of 10% hematoxylin in 95% alcohol. Solution B: Ferric ammonium sulfate (violet crystals), 4.0 g.; glacial acetic acid, 1.0 ml.; concentrated sulfuric acid (sp. gr. 1.8), 0.12 ml.; distilled water, 100 ml. Mix equal parts of Solution A and Solution B; allow to stand overnight, filter and use. For maximum length of staining life, store in full, air-tight bottles. To stain fecal smears, fix in Schaudinn's, pass through iodine alcohol to 50% alcohol, stain for three minutes, wash in running tap water 5 to 15 minutes, dehydrate and mount.**

INTRODUCTION

In attempting to diagnose intestinal protozoa occurring in feces, it is occasionally found that the direct saline preparation and other temporary mounts are inadequate for the identification of some of the observed organisms. In such cases it may be desirable to fix and stain fecal smears in order to render more visible the morphological features of the various intestinal protozoa. Heidenhain's iron-alum hematoxylin technic is generally recognized as the best stain for this purpose. However, many laboratories working under pressure are prevented from using stained smears for diagnosis because the Heidenhain method is lengthy, and because, in order to obtain proper differentiation, each slide must be handled separately during the destaining process.

To overcome these difficulties, various staining technics have been recommended that do not involve controlled destaining. These have usually employed an alum-hematoxylin like Mayer's solution (Craig, 1942, p. 52) or some hematoxylin-phosphotungstic-acid method as described by Dobell (1942), Ratcliffe and Parkins (1944) and Tompkins and Miller (1947). Iron-hematoxylin stains in the

[1]With the technical assistance of Sadie A. Johnson

form of combined mordant and stain mixtures do not seem to have had wide use in the staining of intestinal protozoa, although Weigert's ferric chloride-hematoxylin has been recommended for this purpose[2] (Thomson and Robertson, 1929, p. 349). Such mixtures are usually less stable than the alum hematoxylins, but the staining effects are often more precise and more nearly permanent.

Other iron-hematoxylin mixtures have been described for the staining of tissue sections in Lee's handbook (1937, p. 155) and by Lillie and Earle (1939), and these may be applicable to fecal smears as well. However, these solutions are not generally stocked by most public health diagnostic laboratories, and their preparation for routine use involves more time than such laboratories can usually spare for the purpose.

The formula to be described here includes, as one component, an acidified ferric alum mordant originally recommended by Lang (1936) for use in the usual, Heidenhain iron-hematoxylin technic. We have found that it may also be used to prepare a single-solution stain that does not require destaining. This fact makes it a convenient reagent for the laboratory that may wish to stain smears either for simple, diagnostic purposes, using the single-solution stain, or for careful, detailed study, using the Heidenhain procedure. The acidification of the mordant with sulfuric and acetic acids improves the precision and clarity with which the single-solution mixture stains intestinal protozoa. The use of the ferric salt alone or in combination with either one of the acids alone does not give as good results as when the solution is prepared as described.

Methods

Two solutions are used in preparing this stain:

Solution A:

A 1% solution of hematoxylin in 95% alcohol. This is prepared by diluting a stock solution of 10% hematoxylin in 95% alcohol. This solution need not be ripened.

Solution B:

Ferric ammonium sulfate-violet crystals (iron alum)	4.0 g.
Glacial acetic acid	1.0 ml.
Conc. sulfuric acid (sp. gr. 1.8)	0.12 ml.
Distilled water	100.00 ml.

[2]H. G. Johnstone (University of California, San Francisco) has proposed an iron-hematoxylin mixture for intestinal protozoa that has been adopted in some diagnostic laboratories (unpublished, personal communication).

For use mix equal parts of Solution A and Solution B. The mixture turns a rich purple and overstains when first prepared. Within a few hours the color changes to dark brown with a purplish cast. The solution should then be filtered and may then be used. If the color continues to change until it is greenish-black, the solution is unsuitable for staining. This is often caused by the stock hematoxylin solution's being too ripe and becoming over-oxidized when mixed with the mordant. A freshly prepared hematoxylin solution should then be used instead.

The following staining schedule is recommended:

(1) Fix smears in Schaudinn's plus 5% acetic acid for 5 minutes at 50°–60° C., or for 1 hour or more at room temperature.
(2) Wash in iodized 70% alcohol for 5 minutes.
(3) Transfer through two changes of 50% alcohol for 3 minutes each.
(4) Stain in above solution for three minutes. This is average time for most smears and may be varied slightly to suit different specimens.
(5) Wash in running tap water for 5 minutes. If permanence is important, 15 to 30 minutes is better.
(6) Dehydrate and clear through alcohols, carbol-xylene, and xylene, about 5 minutes each.
(7) Mount in Clarite or other neutral mounting medium.

Due to the oxidizing influence of the ferric salt used in its preparation the stain lasts about a week when kept in open Coplin jars. When stored in completely filled, glass-stoppered bottles the effective life of the stain is extended to several months. If only a few smears are to be stained at irregular intervals, it is practical to stain them individually on a staining rack. Each slide may then be flooded with only a small amount of solution leaving the rest of the stain in the bottle.

We have found that by replenishing the bottled stain after it has been used a few times, with a small amount of freshly prepared stain, that the entire solution will keep longer and stain more vigorously.

Results

The morphology of the intestinal protozoa stained by this method is the same as that shown by the Heidenhain technic. Ordinarily, the cytoplasm of the organisms will stain more darkly and not as transparently as with the best regressive procedures; in addition the fecal matrix of bacteria and debris is stained less precisely. In the case of *Endamoeba coli* cysts it will occasionally be found that some of

the cysts are refractory and do not take the stain. In those instances one of the regressive technics is usually indicated. In the great majority of specimens, however, nuclei, chromatoid bars, fibrils and other diagnostic features of the various intestinal protozoa are well stained and easily differentiated. When first used the solution stains smears a bright blue. As the solution becomes older, the smears stain progressively grayer.

Summary

(1) The preparation and use of a single solution, iron-hematoxylin stain for fecal smears is described.

(2) The stain is conveniently prepared from stock solutions used in a slightly modified Heidenhain technic.

(3) Intestinal protozoa are easily stained with this method and their diagnosis facilitated.

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THE "DISPERSION STAINING" METHOD FOR THE SELECTIVE COLORATION OF TISSUE

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ABSTRACT.—**A method has been developed for the selective coloration of fixed tissue without the use of dyes. Microtome sections of formalin-fixed material are mounted under a cover glass in a mixture of two liquids such as diethylene glycol monobutyl ether with cinnamaldehyde and examined with the dark-field microscope. The refractive index of the liquid used for mounting must be of high dispersion and equal or close to the index of the specimen.**

Tissue elements, dependent on their refractive index, whether slightly lower, the same as, or slightly above the mounting medium appear colored in shades of blue, red or yellow.

The optical principles involved in this optical dispersion method are similar to those involved in the production of colored light by the Christiansen filter.

Histological preparations previous to microscopic examination are usually stained by one of the following methods: (1) vital staining, (2) supravital staining, (3) staining after fixation. Vital staining applies to the specific coloration of certain constituents of living tissue chiefly of the reticuloendothelial system and is probably a simple process of phagocytosis of small particles including those of colloidal size such as lampblack, carmine and trypan blue or the filling of vacuoles with particles of colored matter. Supravital staining is the coloring of tissue elements with non-toxic dyes immediately after removal from the body, such as the staining of mitochondria with Janus green and the neutrophilic, basophilic and eosinophilic granules of leucocytes with neutral red. Staining after fixation is the coloration with dyes of protoplasm following its coagulation, precipitation or chemical combination with fixatives such as formaldehyde, ethyl alcohol, picric acid etc. or with mixtures of these chemicals in correct proportion to best preserve both nuclear and cytoplasmic elements. The mechanism of supravital staining and staining after fixation is usually explained as a process of absorption, adsorption or chemical combination between certain tissue elements and dyes. Specific examples can be given to illustrate each of these theories but most

staining is probably a combination of all three, accompanied by such forces as capillarity and osmosis.

It is the purpose of this paper to present more detailed information concerning another method (Crossmon, 1948) for the coloration of protoplasm without the use of dyes by making use of the refractive indices of the fixed tissue. The technic described is based on the use of the dark-field microscope for the examination of tissue mounted in a liquid equal to or close to its refractive index for sodium light. As observed with dark-field illumination, tissue elements differing widely in index from the mounting medium appear white due to the refracted and reflected white light entering the microscope objective. If the difference between the element and immersion liquid is small, certain portions of their dispersion curves will approach coincidence and light of the corresponding wave lengths will be transmitted straight through or slightly refracted oblique to the optic axis of the microscope and thus not enter the objective. The remaining light for which the object and immersion liquid differ to a greater extent in index is refracted and reflected into the objective. The eye then sees the tissue element as colored, the color being the result of the subtraction of the wave lengths passing straight through or slightly refracted from white light. The method is as follows:

1. *Fixation of Tissue.* Ten per cent neutral formalin in 0.85% NaCl was used since it is best that the fixative employed adds no color to the tissue elements as would be the case with mixtures containing picric, chromic or osmic acid.
2. *Dehydration, Embedding and Cutting.* The alcohol-anilin-oil method of dehydration and clearing followed by xylene previous to paraffin infiltration was used. Tissue was transferred through three changes of 50–52°C. paraffin over a period of approximately 18 hours and sections cut at 12μ.
3. *Affixing Sections.* The usual affixing methods with Mayer's albumen, gelatin or celloidin should not be used since these materials microscopically appear white or colored dependent on the refractive index fluid used for mounting. Sections should be made to adhere to perfectly clean slides without the intervention of any cementing substance, being brought into contact with the glass by evaporation of the distilled water on which they are floated.
4. *Mounting Media.* The mounting media were chosen from a series of liquids differing in index by 0.004 that were equal to or close to the index of the material to be examined and had a much greater dispersion. For best results the liquid should

have low vapor pressure, little or no color, and as applied to tissue preparations, miscibility with xylene. Mixtures of diethylene glycol monobutyl ether and α-chloronapthalene (index range 1.440 to 1.628), α-chloronapthalene and α-bromonapthalene (index range 1.632 to 1.656) and α-bromonapthalene with methylene iodide (index range 1.660 to 1.70) have been used with success for the examination of many mineral and chemical preparations but have not proved to be ideal for most tissue work. Mixtures of diethylene glycol monobutyl ether (Eastman Kodak Co.) with cinnamaldehyde (Eastman Kodak Co.), being of higher dispersion, were found to be much superior for histological preparations especially for the production of strong coloration when using the higher magnifications such as the 43× achromatic objective. The quantity of the lower index liquid to be mixed with the higher index liquid to give the desired intermediate index can be roughly determined by the equation

$$V_1n_1+V_2n_2=V_xn_x$$

where V represents volume, n the index and V_xn_x the volume and index desired. For accurate results it is necessary that the index liquids be checked with a refractometer equipped with temperature regulation apparatus at 25°C. and corrections made by adding the necessary quantity of the lower or higher index liquid.

5. *Equipment.* The microscope and accessories are similar to those described in a previous publication (Crossmon and Gallasch, 1947). The table below gives the optical combinations that have proved successful with a biological type microscope for the examination of microtome sections of histological material. Somewhat inferior results were obtained when using the 10× or 43× achromatic objectives with the Abbe condenser.

Objectives	*Condenser*	*Stop Size*
10× apochromatic (16 mm.), 0.30 N.A.	Achromatic, 1.40 N.A. Top element removed to give 0.59 N.A.	13–14 mm.
20× achromatic (10.25 mm.) 0.40 N.A.	As above.	16–18 mm.
43× achromatic (4mm.), 0.65 N.A. with funnel stop.	As above.	16–18 mm.
10× achromatic (16 mm.) 0.25 N.A.	Abbé, 1.25 N.A. Top element removed to give 0.30 N.A.	16–17 mm.
43× achromatic (4mm.), 0.65 N.A. with funnel stop.	As above.	22 mm.

The light source should be very bright. A research microscope lamp having a 6 volt 108 watt ribbon filament bulb is satisfactory. For best results it is important that a daylight glass, 50 mm. square, polished on both sides for maximum transmission be used as a filter and that the lamp be equipped with an iris diaphragm.

6. *Microscopic Examination.* Previous to examination with the microscope, the microtome section should be placed in two or three changes of dust-free xylene to dissolve the paraffin completely. The xylene surrounding the tissue should be wiped away with a lint-free cloth and just before the section is entirely dry it should be covered with the desired index fluid and a cover glass applied. The preparation is now ready to examine using first a 10× compensating eyepiece in conjunction with the 10× apochromatic objective. The upper element of the achromatic condenser should be removed, thus reducing its numerical aperture to 0.59 and a dark-field stop 13 to 14 mm. should be placed in the stop holder below the iris diaphragm. It is important that the plane microscope mirror be used and that the microscope lamp be adjusted to produce a parallel beam of light. The condenser should now be racked up or down as may be necessary until the preparation appears bright on a dark background. Increased magnification can be obtained by substituting 15× or 25× compensating eyepieces for the 10× or the use of the 20× and 43× objectives. If the 43× is used, its numerical aperture must be reduced by the insertion of a funnel stop screwed into the objective far enough as to just touch the back lens. A funnel stop with overall length of 1.092 inches and aperture of 0.116 inches has proved to be satisfactory for most preparations.

It is important that the lamp iris diaphragm be carefully adjusted especially when using the 43× objective to prevent internal reflections which often cause a haze over the field of view. It should be partially closed and then opened until it just clears the field of the eyepiece.

Results and Discussion

The results obtained are dependent on the refractive index of the tissue elements examined and the liquid used as the mounting medium. Provided the index of the tissue and fluid differ greatly, the microtome section will appear white on a dark background as is commonly observed with dark-field illumination. However, if

the index fluid has a much higher dispersion than the tissue, as is usually the case, and is close to it in refractive index, those elements formerly appearing white will be colored blue, red or yellow dependens on their index, whether slightly lower, the same as or slightly above the mounting medium used.

The range of refractive index liquids suggested in this paper is much lower and higher than will be commonly necessary for histological preparations fixed in 10% neutral formalin. It is probable that index liquids slightly above or below 1.536 prepared from mixtures of diethyl glycol monobutyl ether with cinnamaldehyde will be of chief use. Increased accuracy can be obtained by adjusting the index liquids mainly used to a difference of 0.002 and examining the preparation microscopically in a temperature controlled room at 25°C.

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CROSSMON, G. C., and GALLASCH, B. G. 1947. Counting of blood cells by dark-field illumination. J. Lab. and Clin. Med., **32**, 206–9.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

COLE, V. W. **Polarized light in photo-microscopy.** *J. Biol. Photo. Assoc.*, **16**, 147–50. 1948.

Polarized light has been used for the examination of anisotropic substances such as the cross striations in muscle, cellular crystallin substances and lipoid material. Since the photography of anisotropic material by use of Nicol prisms is somewhat complicated and expensive, the author suggests the substitution of polaroid lenses. One lens, the analyzer, is placed over the eyepiece of the microscope and the other, the polarizer, incorporated in the light source. A microscope having an apochromatic objective lens (3 mm., N.A. 0.95) and a 10× compensating eyepiece used in conjunction with a carbon arc light proved to be the most efficient combination. Two different cameras are suggested although any camera with a ground glass back is adaptable. Comparison photographs of motor end plates (gold chloride stain) with and without polaroid lens are shown.—*G. C. Crossmon.*

CROSSMAN, GERMAIN. **The counting of red blood cells by dark-field illumination.** *J. Lab. & Clin. Med.*, **32**, 206–9. 1947.

An ordinary binocular microscope can be readily fitted for dark-field illumination and used for counting blood cells, cells in spinal fluid, bacteria, yeasts, molds, plankton, and dust. For blood cells the following procedure is recommended: Dilute as usual and place in a hemocytometer. Adjust a 6 volt 108 watt bulb to give a parallel beam by focusing the image of the bulb on the wall at 6 feet. Use a Corning Daylite Filter. Place a 16 mm. dark-field stop below the condenser. Use a 10×, 0.25× N.A. low power objective and 10× or 15× oculars. Rack the substage condenser until the ruling and the cells appear white on a dark background. A 43×, 0.65× N.A. objective may be used but an approximately 20 mm. funnell stop is then required. Rheinberg color discs may be substituted for the opaque stops, but have no real advantage, although the appearance is spectacular.—*John T. Myers.*

PEASE, D. C., and BAKER, R. F. **Sectioning techniques for electron microscopy using a conventional microtome.** *Proc. Soc. Exp. Biol and Med.*, **67**, 470–4. 1948.

A method for cutting 0.2μ sections was developed for a standard Spencer rotary microtome. Rat liver fixed by perfusion with 2% osmic acid was doubly embedded with Parlodion and paraffin. The face of the block to be cut was pared down to about 1 mm. square. The microtome was modified by adding a low angle incline to the feed mechanism to give an advance of only 1/10 the calibrated value. The technic is illustrated, and the original article should be consulted for many essential details.—*H. A. Davenport.*

RICHARDS, OSCAR W. **Phase photomicrography.** *J. Biol. Photo. Assoc.*, **16**, 29–38. 1947.

The phase microscope shows organisms that are transparent or that have low contrast in sharp outline and with internal detail at full resolution. Instructions are given for use of this instrument for photomicrography. Methods of focusing, the use of a fast flash-tube light for stopping motion in the specimen, and a simple visual step photometer for estimating correct exposure are described. The advantages of stereophotomicrographs made from pictures taken with the phase microscope are discussed, and methods of making them are given.—*Elbert C. Cole.*

TAYLOR, E. WILFRED. **The practical application of phase-contrast to the biologist's microscope.** *J. Roy. Micr. Soc.*, **66**, 1–8. 1946.

This article is an excellent account of the phase-contrast principle in microscopy. The introduction states clearly the use to which the biologist will put this new accessory. The historical account is complete in dealing with the discovery and later modifications. A very accurate description of the method as applied to the microscope is given. An adequate presentation of the theory strengthens the article considerably. The final portion deals with the adjustments necessary in the use of phase-contrast. Five plates and six text figures illustrate the use and results obtained by phase-contrast as used in biological work.—*Paul G. Roofe.*

MICROTECHNIC IN GENERAL

DAVIS, H. L., and RYNKIEWICZ, H. J. **A fiber identification stain.** *Ind. Eng. Chem., Anal. Ed.*, **14**, 472. 1942.

The dye mixture contains: acid fuchsin (Color Index No. 692), 6 g.; picric acid, 10 g.; tannic acid, 10 g.; and National soluble blue 2B extra (Color Index No. 707), 5 g. The dyes may be ground together and dissolved in hot water or dissolved separately in any order and diluted to 1 liter. Momentary immersion of fibers in the hot solution gives adequate staining, but 2 or more minutes are required for cold staining. A thorough rinsing in water completes the test. Some dyed textiles may be identified without previous bleaching. When pressed wet (after rinsing) between white absorbent papers, a dye mixture characteristic of the color which would have been shown by the undyed fibers is transferred to the papers. Colors shown by common fibers are: cotton or linen, light blue; wool, yellow; raw silk, black; degummed silk, brown; acetate or nylon, pale greenish yellow; cuprammonium, dark blue; viscose, lavender; vinyon, very pale blue. A solution of this dye mixture is made available by Eimer and Amend, New York, N. Y.—*R. T. Whittenberger.*

NICHOLS, J. **Improved method for handling photolabile liquids.** *Arch. Path.*, **44**, 646–7. 1947.

Disadvantages of present methods of storing liquids such as silver nitrate and osmic acid are discussed. A new device, consisting in placing a properly prepared bottle in a black cup, is described. The bottle is first painted black except for a portion at the base somewhat less than that which will be covered by the cup. Then when the bottle is lifted from the cup, the state of the liquid can be determined readily. The flat-bottomed cup need not be removed during pouring.—*Warren Andrew.*

ANIMAL MICROTECHNIC

GRIGGS, FREDERICK C. **The chromosome smear technique: a critical review and improvement of method.** *J. Roy. Micr. Soc.*, **66**, 25–33. 1946.

The following four sections are presented in detail: (1) nature of the problem; (2) problems involved in modern methods; (3) requirements of an efficient technic; (4) consideration of results.

Modern cytological problems are presented in the first two areas. The third section is outlined as follows: A. Reasonably rapid procedure is necessary. B. The technic must be of such a nature that no great loss of material occurs. C. Transparency is stressed. D. Internal optical differentiation is emphasized. E. The stain must give sharp details. F. The technic must not allow changes of any consequence in preparation after immersion in fluid balsam.

The fourth portion goes into specific consideration of results by the author's own technic which is given below:

(1) Smear in the usual way with fresh material and allow to desiccate for a few seconds (blowing assists this process).

(2) Invert slide over following pre-fixative: 80% alcohol, 80 vol.; glacial acetic acid, 20 vol.

Allow to act for 20–30 min. according to material.

(3) Drain and flood with fresh stain-fixative which consists of *Solution A:*

0.5% hematoxylin (about 2–3 weeks old) 1 vol.; 4.0% iron alum (fresh) 0.75 to 1 vol. (or less); absolute alcohol 2 vol., glacial acetic acid 0.5 to 0.75 vol.; with an equal quantity of *Solution B:* 12.5–20% aqueous acetic acid plus carmine to saturation, boiled and filtered. (Concentration of acetic should be approximately near to that of solution A.)

(4) Wash in 50%, then 80%, and finally 100% alcohol, taking care to ensure that the acetic acid is completely cleared from the slide.

(5) At least three changes of alcohol/xylene should be used in ascending concentrations of xylene; but it is undesirable to pass into pure xylene so one should mount from about 60% xylene plus 40% alcohol. Solution B can be added in a lower proportion than 50% to the stain-fixative (3), in which case the concentration of alcohol goes up. Stage 2 should be so varied that stages 2 and 3 together are completed in about 25–35 min.; some material appears to require more pre-fixation and less staining than others, but the above schedule has been used to produce the slides from which the photomicrographs were taken, and can profitably be considered a starting point for most work. The stain should be used within 5–6 hours of making up because a precipitate occurs and staining capacity is lost on filtration.

The author's concluding remarks are as follow: "This technic has not proved easy to handle but, when good preparations are obtained, inner detail of chromosomes is revealed in a manner unobtainable by any other method."—*Paul G. Roofe.*

HSU, J. H. **An improved method for counting blood platelets.** *Science and Technology in China,* **1,** 54–5. 1948.

It was found that by using Kristenson's method for counting blood platelets, the platelets form clumps, unevenly distributed; or the individuals may enlarge in size and shape so as to become indistinguishable from a clump of aggregated platelets. The author suggests a solution designated as U.C.F. which avoids these defects. The formula is 40% formaldehyde, 0.1 ml.; urea , 10 g.; sodium citrate, 0.5 g.; distilled water, 100 ml. The red cells are completely destroyed, and the platelets are well preserved, no change in shape and size nor in number being detected within one hour after dilution. The platelet count is performed in the usual manner except for the use of a white blood pipette in which blood, obtained from a finger puncture, is diluted 20 times with the fluid. Eighty small squares in the red area are counted and multiplied by 1,000 to give the number of platelets per cubic milimeter.—*M. A. Darrow.*

KRAMER, SOL. **A staining procedure for the study of insect musculature.** *Science,* **108,** 141–2. 1948.

A method for in toto muscle differentiation in housefly larvae and similar forms is described. Procedure is as follows: Bouin's fluid (30°) 8–10 hr.; 50% ethyl alcohol, 10 min.; 70% ethyl alcohol, 1 hr.; 95% ethyl alcohol, 10 min.; 0.5% eosin alcohol, 6–8 hr.; return to 95% alcohol and add oil of wintergreen dropwise to larvae in 95% alcohol at hourly intervals over a period of 4–5 days; transfer to oil of wintergreen. Details of procedure and methods of examination are given in the discussion.—*T. M. McMillion.*

LAECOM, RODNEY C., and CARTER, GEORGE H. **Erythrocytes in urinary sediment: identification and normal limits, with a note on the nature of granular casts.** *J. Lab. & Clin. Med.,* **33,** 875–80. 1948.

The erythrocytes are stained by the following procedure:

Place a drop of sediment on a slide and add a drop of benzidine-nitroprusside solution made by diluting 1.0 ml. of 1.0% aqueous benzidine with 6.0 ml. of water and adding 1.0 ml. of 2% aqueous sodium nitroprusside (stable for about 2 weeks). Mix by tilting. Add 1 drop of 3% H_2O_2 and mix. If the urine is alkaline, add 1 or 2 drops of 10% nitric acid to the sediment in a centrifuge tube, wash 3 times with 0.9% NaCl solution and proceed as above. Erythrocytes are stained a deep blue-purple. If the cell has deteriorated, it will stain light blue. Yeast cells show a blue-green irridescence. Leucocytes have a pale gray cytoplasm and a slightly darker irregularly stained nucleus. The granules of casts have the bluish purple hue of erythrocytes. Fungi and spores, pollen grains, and prostatic bodies

are not stained. The stain is based on the pseudoperoxidase reaction of hemoglobin and benzidine. The nitroprusside reagent is a catalyst. Erythrocytes may occur in normal urine up to 2 per high power field. Formaldehyde is a satisfactory preserving agent.—*John T. Myers.*

STRUGGER, S. **Fluorescence microscope examination of trypanosomes in blood.** *Canad. J. Res.*, Sec. E, **26**, 229–31. 1948.

To stain vitally living trypanosomes in blood the following procedure is recommended: Mix a drop of freshly-taken blood with a 1:10,000 solution of acridine orange (3,6-tetramethyldiaminoacridin) made with 0.85% sodium chloride; cover with cover slip; examine with a blue light fluorescence microscope. This microscope may be constructed as follows: for light use a carbon-arc lamp with attached convex lens to produce parallel rays: filter light with a cuvette (2½ cm. thick) filled with a saturated solution of copper oxide ammonia so that only blue light reaches the plane mirror of the microscope; over ocular, insert filter containing an orange glass that absorbs blue light quantitatively but allows green, yellow, and red to pass through unchanged; to focus, use slide on which is placed pulverized anthracene in liquid paraffin. Leucocytes and trypanosomes are visible as motile shining, bright green bodies but the erythrocytes are nonfluorescent and scarcely visible. To stain trypanosomes in dried blood smears: smear blood; dry; fix 2 to 3 min. in methyl alcohol; wash; stain 4 min. in auramine solution, 1 part in 1000 parts distilled water, with 5% liquid phenol added later; wash in distilled water 1 or 2 min.; dry in air; keep slide in darkness. With blue light fluorescence microscope, background is black, erythrocytes shine slightly as dark green circles, and trypanosomes shine with bright golden fluorescence. Contrast is improved if a drop of liquid paraffin and a cover slip are put on stained smear.—*Herbert P. Riley.*

PLANT MICROTECHNIC

FLINT, THOMAS J., and MATZKE, EDWIN B. **Recent procedures for the study of three-dimensional shapes of resting cells, and a new method for the shape study of cells in division.** *Science*, **108**, 191–2. 1948.

Stem tips of *Anacharis densa* (Elodea) were dissected out, killed in CRAF fixative, embedded, and sectioned at 25μ or thicker. They were then stained with the Feulgen technic as outlined by de Tomasi, except that they were hydrolyzed longer (30 min.) in *N* HCl and stained in decolorized fuchsin for 24 hr. After being rinsed in distilled water the slides were put in a strong aqueous ruthenium red solution for 30 min., quickly rinsed again in distilled water, and run as rapidly as possible into absolute alcohol. They were then stained 1½ min. in orange G in absolute alcohol and clove oil, rinsed in clove oil, put in xylene, and mounted in neutral balsam.

The results were as follows: chromatic figures of dividing cells, deep purple; resting nuclei, less intense; middle lamellae darkened by ruthemium red and spindle fibers and cell plate stained by orange G; cellulose walls, deeply colored.—*T. M. McMillion.*

HITIER, H., and QUIDET, P. **Obtention de plantes polyploides par traitement a l'hexachlorocyclohexane et au sulfure de polychlorocyclane.** *Compt. Rend. Acad. Sci. Paris*, **226**, 833. 1948.

Changes in chromosome number similar to those induced by colchicine were observed in tobacco and onion after treatment with hexachlorocyclohexane and polychlorocyclane sulfate under the following conditions: 8% powdered hexachlorocyclohexan, tobacco, 3 to 4 leaves; 2% powdered hexachlorocyclohexan, tobacco, 8 to 10 leaves; 0.01% powdered hexachlorocyclohexan, roots, onion; 8% powdered hexachlorocyclohexan, base of onion bulb; 0.045% sulfur of polychlorocyclane, tobacco, 8 to 10 leaves; 0.05% sulfur of polychlorocyclane, roots, onion; 14% powdered sulfur of polychlorocyclane, base of onion bulb.—*T. E. Weier.*

MICROÖRGANISMS

HUNTER, LILLIAN M. **A study of the mycelium and haustoria of the rusts of Abies.** *Canad. J. Res.*, Sec. C, **26**, 219–38. 1948.

Infected leaves of Abies were examined for mycelia and haustoria of certain rusts using fixatives previously used in a study of spermogonia. Because of the difficulty of showing clearly the entrance of the haustorium of *Milesia polypodophila* into the cells of the cortex of the stem, the following procedure was substituted: fix infected stems with leaves attached in medium chrom-acetic solution; treat in usual manner and embed in celloidin; section at 7 μ to 15μ on a sliding microtome; take sections through usual grades of alcohols, dissolve in celloidin, and lead to a lactophenol solution in graded solutions from 95% or 70% alcohol; prepare solutions of acid fuchsin, cotton blue, and light green, dissolved separately in lactophenol with just enough stain to give a deep color; drain lactophenol from sections and apply each stain separately by a pipette; cover sections with bell jar and leave overnight; the next morning, wash in lactophenol solution to remove stain; mount with cover glass and a few days later ring with amber cement. The haustoria were clearer than with any other method of staining used. The value of the method was in demonstrating the penetration by the mycelium of the wall of the host cell.—*Herbert P. Riley.*

LEVINE, B. S., and BLACK, L. A. **Newly proposed staining formulas for the direct microscopic examination of milk.** *Amer. J. Pub. Health*, **38**, 1210–18. 1948.

A theoretical discussion is first presented of certain principles of adsorption as they apply to the study of staining procedures for the direct microscopic examination of milk.

Based on the above principles, the authors have developed a two-dip and a one-dip procedure for defatting and staining milk films. The two-dip method consists in defatting films as usual (Standard Methods for the Examination of Dairy Products, *8th Ed*), followed by a one-minute submersion of the slide in a 0.6% solution of methylene blue in 95% alcohol. Their one-dip, water- and acid-free solution consists of certified methylene blue powder, 0.6 g.; ethyl alcohol, 95%, 50.0 ml.; tetrachlorethane, technical, 50.0 ml.; glycerol, C.P., 2.5 ml. Slides are immersed in the solution for one minute.

The above staining solutions yielded maximal bacterial counts of milk, as compared with carbolated methylene blue or the Newman-Tampert stain (Standard Methods, *8th Ed.*). A polychrome methylene blue solution yielded materially higher counts than carbolated methylene blue.—*M. W. Jennison.*

STRUGGER, S. **Fluorescence microscope examination of bacteria in soil,** *Canad. J. Res.*, Sec. C, **26**, 188–93. 1948.

To observe living soil bacteria in their native condition, soil was stained with the fluorescent dye, acridine orange (3,6-tetramethyldiaminoacridin). If living cells are stained with this dye (solution 1:10,000, pH 7.5) the living cytoplasm and the nucleus show green fluorescence, but if dead cells are stained the dead protoplasm develops a bright copper-colored fluorescence. The staining of soil is carried out as follows: place 1 g. of sifted soil in each of five test tubes; to each tube add 10 ml. acridine orange solution made with tap or spring water; shake. As soils differ in ability to absorb stain, the same soil must be stained with five different concentrations of acridine orange (1:1000, 1:2000, 1:3000, 1:4000, 1:5000). The most suitable concentration can be determined: it is satisfactory when only a little excess stain remains in the solution after heavy shaking. Generally the soil is stained completely in a few minutes, but it is advisable to wait 5 or 10 min. before observing with the fluorescent microscope. Two methods of preparing slides are used: (1) for qualitative analysis of autochthonic bacteria, a stained soil suspension is strongly centrifuged, the solution on top is decanted, and a small quantity of sediment is well mixed in drop of paraffin oil on a slide and the drop is covered with a cover slip; (2) for counts, a small drop is taken with a needle from the stained and shaken soil suspension, put on a slide, and covered with a cover slip. Examination is by a luminous blue light fluorescence microscope with an oil immersion lens. Paraffin oil is a good immersion fluid.

For counting, a counter of 20μ depth combined with a counting-ruled ocular is used. The particles of soil covered with humus and the particles of humus slime have a dim red fluorescence and the living bacteria are green. The stained bacteria are not killed and can be used for culture experiments.—*H. P. Riley.*

HISTOCHEMISTRY

JAQUES, LOUIS B., BRUCE-MITFORD, MARGARET, and RICKER, ANN G. **The metachromatic activity of heparin.** *Rev. Canad. Biol.*, **6**, 740–54. 1947.

When heparin is added to many dyes containing a free amino group, it causes a change in the color of the dye, shifting its absorption spectrum to a shorter wave length. When 2 mg. of heparin are added to 5 ml. of a 5 mg. % solution of bismark brown, the color changes from orange to yellow. With azure A the change is from blue to red, with brilliant cresyl blue or brom cresyl blue from blue to purple, with Nile blue sulfate from greenish blue to reddish blue, and with neutral red at pH 6.7 from red to orange. With basic fuchsin there is a detectable shift in the red color, and with pyronin and acriflavin the solution loses its fluorescence. These color changes are termed metachromatic reactions.

To determine the metachromatic activity of heparin, the following method was used, based on the measurement in the Lovibond tintometer of the red color developed with azure A (National Aniline Corp. certified stain with dye content 61%): Make 100 mg. % azure A and keep as a stock solution in the refrigerator. Just before use make a 25 mg. % solution from the stock solution. Make $M/10$ borate buffer solution (pH 9.8) and dissolve enough sodium chloride in it to give 0.85%. Use, as standard heparin, crystallin barium salt or sodium salt of heparin. Dissolve unknown sample in physiological saline containing 0.3% tricresol and adjust pH to 9–10 if very strongly buffered. To 2 ml. of buffer-heparin sample add saline to 4 ml. Add 1 ml. of dye solution, mix, transfer to cell of tintometer, and match color quickly, using red and blue glasses. Construct the standard curve by carrying out the procedure with known amounts of standard heparin and plotting the resulting red readings. Dilute unknown solutions until they give a reading in the steep part of the curve, and test further dilutions to show that they result in lower readings.

Heparin isolated as the crystallin barium salt from different species and beef heparin partly inactivated by storage in acid solution at 5° C. for 18 months showed the same metachromatic activity. Since these various sources showed different anticoagulent activity, apparently the measurement of metachromatic activity measures heparin present on a weight basis irrespective of the factors responsible for its anticoagulant activity. If metachromatic measurements are to be made, Lloyd's reagent, often used to purify heparin, must be used with great caution if at all, because it is very strongly metachromatic.—*H. P. Riley.*

LIVINGSTON, R., and PARISER, R. **The chlorophyll-sensitized photo-oxidation of phenylhydrazine by methyl red. II. Reactivity of the several forms of methyl red.** *J. Amer. Chem. Soc.*, **70**, 1510–5. 1948.

Methyl red can exist in three colored forms in methanol, the relative concentration depending upon the acidity of the solution. In the chlorophyll-sensitized photo-oxidation of phenylhydrazine, only the intermediate form of methyl red reacts. The quantum yield is an empirical function of the stoichiometric concentration of phenylhydrazine and of the concentration of the intermediate form of methyl red. Over a fairly wide range the quantum yield is independent of the light intensity and of the concentration of chlorophyll. Further measurements of this type may be of aid to the understanding of the "inner mechanism" of photosynthesis.—*R. T. Whittenberger.*